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NGN3 Expression in Definitive Endoderm Progenitors Specifies Pancreatic Endocrine Precursors

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Graduate Program in Physiology
A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science
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**NGN3 EXPRESSION IN DEFINITIVE ENDODERM PROGENITORS
SPECIFIES PANCREATIC ENDOCRINE PRECURSORS**

(Thesis Format: Monograph)

By

Ahmed Shah

Graduate Program

in

Physiology and Pharmacology

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

The School of Graduate and Postdoctoral Studies

University of Western Ontario

London, Ontario, Canada.

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Abstract

Our lab previously demonstrated that expression of the lineage-determining transcription factor SOX17 in human embryonic stem cells was sufficient to specify stable definitive endoderm progenitors (DEPs). The current study was aimed at generating pancreatic precursors from SOX17-DEPs through controlled expression of the transcription factor NGN3. We generated hESC lines with inducible SOX17 and NGN3 and compared the effects of high and low levels of NGN3 expression. NGN3 expression in DEPs induced markers of pancreatic differentiation (PAX4, PAX6, NKX6.1, ISL1). While high NGN3 expression induced the β cell marker PDX1, low NGN3 expression induced the δ cell markers (CCK_BR, somatostatin) within 12 days. We subsequently aimed to guide SOX17-NGN3 precursors to mature endocrine cells by culture in media pre-conditioned by multipotent stromal cells previously screened for islet regenerative capacity following transplantation *in vivo*. However, endocrine maturation was not observed. These findings demonstrate the precision required to direct β cell differentiation.

Keywords

human embryonic stem cells, SOX17, NGN3, pancreatic progenitors, multipotent stromal cells

Co-Authorship Statement

The cloning of PB-NGN3 transgene vector, the generation of SOX17; PB-NGN3 clones used in this study, and the experiments described in chapter 2 were performed by Ahmed Shah in the laboratories of Drs. Cheryle Séguin and David Hess. For all experiments Drs. Séguin and Hess provided assistance in experimental design and data interpretation. Furthermore, Drs. Séguin and Hess assisted in manuscript preparation by proofreading, editing, and providing critical feedback. Courtney Brooks assisted in the maintenance of SOX17; PB-NGN3 cell cultures. John Krakovsky performed real-time PCR experiment shown in Figure 3.4A of this thesis. Stephen Elliot Sherman performed the *in vivo* characterization of regenerative and non-regenerative bone-marrow derived MSCs used in this study.

Dedication

*I dedicate this thesis to my girlfriend Fitore, for her constant support and encouragement
in the difficult period before and after my surgery and treatment.*

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I would like to thank my supervisors Dr. Cheryle Séguin and Dr. David Hess for their ongoing supportive attitude for my project and my future endeavors during these two years. I am grateful for current members of Séguin Lab, especially Courtney Brooks for her training, constant help and mentorship. I would also like to thank Michael Sattin and Nicole Watts, former students of Seguin lab, for their prompt responses to my inquiries and also showing their interest in the project's progress despite living a few hundred kilometers away from London, ON. I am very thankful to my girlfriend and my family for their strong support during the past two years.

TABLE OF CONTENTS

Title Page	i
Abstract	ii
Keywords	ii
Co-Authorship Statement	iii
Dedication	iv
Acknowledgements	v
Table of Contents	vi
List of Tables	x
List of Figures	xi
List of Appendices	xiv
List of Abbreviations, Symbols, Nomenclature	xv

CHAPTER 1: General Introduction	1
1.1. Diabetes Mellitus	2
1.1.1. Types of Diabetes Mellitus	2
1.2. Pancreas Biology	4
1.2.1. The Exocrine Pancreas	4
1.2.2. The Endocrine Pancreas	4
1.2.2.1. α cells	5
1.2.2.2. β cells	7
1.2.2.3. δ cells	8
1.2.2.4. F cells	10

1.2.2.5. ϵ cells	11
1.3. Pancreatic Development	11
1.3.1. Formation of the Pancreas from Definitive Endoderm	12
1.3.2. Role of Transcription Factors in Pancreatic Endocrine Development	13
1.3.2.1. SOX17	14
1.3.2.2. Early Pancreatic Transcription Factors ((HNF1 β , HNF6, PDX1, SOX9 and PTF1a)	14
1.3.2.3. NGN3	15
1.3.2.4. Additional Pancreatic Endocrine Transcription Factors	16
1.4. Stem Cells	19
1.4.1. Human Embryonic Stem Cells (hESC)	20
1.4.2. Human Bone-Marrow Derived Multipotent Stromal Cells (MSC)	20
1.5. Cell-based therapies for Diabetes Mellitus	21
1.5.1. Islet Transplantation	21
1.5.2. Differentiation of Embryonic Stem Cells to Islet Cells	22
1.5.2.1. Growth Factor Based Differentiation	22
1.5.2.2. Transcription Factor Based Differentiation	24
1.5.3. Islet regeneration mediated by Human MSCs	25
1.6. Strategies for Transcription Factor Based Differentiation of hESC	27
1.6.1. SOX-17 ER ^{T2} System	27

1.6.2. NGN3 piggybac System	28
1.6.3. Strategies for Enhancing Maturation of Endocrine Precursors Cells	28
1.7. Rationale	29
1.8. Hypothesis	30
1.9. Objectives	30
CHAPTER 2: Materials and Methods	31
2.1. Generation NGN3 expression constructs	32
2.2. Generation of transgenic SOX17-ER ^{T2} ; PB-NGN3 inducible cell lines	32
2.3. Cell culture	34
2.4. Generation of MSC Conditioned Media (CM)	35
2.5. Quantification of PANC-1 cell proliferation	36
2.6. Gene expression analysis	36
2.7. Immunostaining	39
2.8. β -galactosidase staining	40
2.9. Statistical analysis	40
CHAPTER 3: Results	41
3.1. Characterization of CA2 SOX17 ^{ERT2} ; PB-NGN3 clonal cell lines	42
3.2. Differentiation of pluripotent hESCs to definitive endoderm progenitors (DEP)	46
3.3. NGN3 expression directs SOX17-DEPs to a pancreatic endocrine	

fate	46
3.4. Development of <i>in vitro</i> strategy to assess the effects of MSC-secreted factors on the differentiation of SOX17-ER ^{T2} ; PB-NGN3 cells	63
3.5. Islet-regenerative MSC-conditioned media promotes PANC-1 cell proliferation <i>in vitro</i>	65
3.6. Culture of SOX17-ER ^{T2} ; PB-NGN3 cells in regenerative MSC-CM does not promote pancreatic endocrine maturation	67
CHAPTER 4: Discussion and Future Directions	73
4.1. NGN3 expression in SOX17-DEPs specifies endocrine cell fate	74
4.2. The level of NGN3 expression in SOX17-DEPs influences endocrine fate determination	77
4.3. Islet-regenerative MSC conditioned media did not promote maturation of NGN3-positive endocrine precursors	79
4.4. Limitations of this study and future directions	81
4.5. Conclusion and summary	82
CHAPTER 5: References	84
Appendix	101
Curriculum Vitae	104

List of Tables

Table 2.1. Primer sequences.	37
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List of Figures

Figure 1.1. Schematic representation of cellular organization and composition of murine and human islets of Langerhans.	6
Figure 1.2. Schematic representation of glucose-mediated insulin secretion in β cells.	9
Figure 1.3. Summary of transcription factor-mediated pancreatic endocrine specification during mammalian development.	18
Figure 2.1. Alignment of human NGN3 coding sequence with PB-NGN3 transgene.	33
Figure 3.1. Characterization of clonal hES cell lines with inducible NGN3 expression.	43
Figure 3.2. Immunolocalization of NGN3.	45
Figure 3.3. Characterization of SOX17-induced definitive endoderm differentiation in SOX17-ER ^{T2} ;PB-NGN3 cell lines.	47
Figure 3.4. SOX17-ER ^{T2} ; PB-NGN3 ^{LOW} and SOX17-ER ^{T2} ; PB-NGN3 ^{HIGH} clones show loss of pluripotency-associated factors following NGN3 induction.	49
Figure 3.5. NGN3 induction in SOX17 DEPs promotes expression of pancreatic endocrine markers dependent on the level of NGN3.	52
Figure 3.6. NGN3 induction in SOX17-ER ^{T2} ; PB-NGN3 ^{LOW} cells induces areas of PAX6 positive cells.	54
Figure 3.7. NGN3 induction in SOX17-ER ^{T2} ; PB-NGN3 ^{LOW} cells induces areas of ISL1 positive cells.	55
Figure 3.8. NGN3 induction in SOX17-ER ^{T2} ; PB-NGN3 ^{HIGH} cells induces areas	

of PAX6 positive cells.	56
Figure 3.9. NGN3 induction in SOX17-ER ^{T2} ; PB-NGN3 ^{HIGH} cells induces areas of ISL1 positive cells.	57
Figure 3.10. SOX17-ER ^{T2} ; PB-NGN3 cells do not express insulin protein after 12 days of NGN3 induction.	59
Figure 3.11. SOX17-ER ^{T2} ; PB-NGN3 ^{LOW} cells do not express SST protein after 12 days of NGN3 induction.	61
Figure 3.12. SOX17-ER ^{T2} ; PB-NGN3 ^{HIGH} cells do not express SST protein after 12 days of NGN3 induction.	62
Figure 3.13. Culture of hESC and SOX17-DEPs in serum-free MSC media does not promote spontaneous differentiation.	64
Figure 3.14. Islet-regenerative MSC-conditioned media promotes PANC-1 cell proliferation.	66
Figure 3.15. Culture of SOX17-ER ^{T2} ; PBNGN3 ^{LOW} cells in regenerative MSC conditioned media (CM) does not promote pancreatic endocrine maturation.	68
Figure 3.16. Culture of SOX17-ER ^{T2} ; PBNGN3 ^{HIGH} cells in regenerative MSC conditioned media (CM) does not promote pancreatic endocrine maturation.	69
Figure 3.17. Culture in the presence of regenerative MSC conditioned media (CM) during later stages NGN3-mediated differentiation of SOX17-ER ^{T2} ; PB-NGN3 ^{LOW} cells enhanced the expression of early pancreatic endocrine markers.	71
Figure 3.18. Culture in the presence of regenerative MSC conditioned media	

(CM) during later stages NGN3-mediated differentiation of SOX17-ER^{T2}; PB-NGN3^{HIGH} cells enhanced the expression of early pancreatic endocrine markers. 72

List of Appendices

Appendix 1. Immunocytochemistry positive control experiments	101
Appendix 2. Immunocytochemistry negative control experiments	103

List of Abbreviations, Symbols, Nomenclature

α	alpha
β	beta
β GEO	beta-galactosidase neomycin fusion protein
β TC6	mouse β cells expressing T-antigen of simian virus 40 (SV40)
δ	delta
ϵ	epsilon
$^{\circ}\text{C}$	degree Celsius
pg	picogram
ng	nanogram
μg	microgram
mg	milligram
g	gram
μL	microliter
mESC	mouse embryonic stem cells
mL	millilitre
mM	millimolar
μm	micromolar
ADP	adenosine diphosphate
AMP	adenosine monophosphate
ANOVA	analysis of variance
ARX	aristaless related homeobox
ATP	adenosine triphosphate

BMP4	bone morphogenic protein 4
Ca ⁺²	calcium ion
CCK _B R	cholecystokinin B receptor
cDNA	complimentary DNA
CER1	cerberus 1
CXCR4	C-X-C chemokine receptor type 4
CYC	cycle
DLX5	distal-less homeobox 5
DMEM	Dulbecco modified Eagle's minimal essential medium
DNA	deoxyribonucleic acid
DOX	doxycycline
e	mouse embryonic day
EML	stem cell factor-dependent mouse lympho-hematopoietic progenitor cells
ER	endoplasmic reticulum
FBS	fetal bovine serum
FGF10	fibroblast growth factor 10
GCG	glucagon
GCK	glucokinase
GH	growth hormone
Glut 2	glucose transporter type 2
GUSB	beta-glucuronidase
h	hour
HNF1 β	hepatocyte nuclear factor 1 homeobox β

HNF6	hepatocyte nuclear factor 6
IGF	insulin-like growth factor
INS	insulin
INSM1	insulinoma associated 1
ISL1	insulin gene enhancer protein
K ⁺	potassium ion
MAFA	V-maf musculoaponeurotic fibrosarcoma oncogene homolog A
mPAC	mouse pancreatic adenocarcinoma cells
MPC	multipotent pancreatic progenitor cells
mPDEC	mouse pancreatic ductal epithelial cells
mRNA	messenger ribonucleic acid
n	sample size
NGN3	neurogenin 3
NKX2.2	NK2 transcription factor related, locus 2
NKX6.1	NK6 homeobox 1
NOG	noggin
PAX4	paired box gene 4
PAX6	paired box gene 6
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PC	proprotein convertase
PDX1	pancreatic duodenal homeobox-1
PECAM	platelet endothelial cell adhesion molecule/CD31

PP	pancreatic polypeptide
PTF1a	Pancreas transcription factor 1 subunit alpha
qPCR	quantitative polymerase chain reaction
RA	retinoic acid
RER	rough endoplasmic reticulum
RFX6	regulatory factor X type 6
RNA	ribonucleic acid
RT	reverse transcribed
RyR3	ryanodine receptor type 3
SB	activin/nodal inhibitor SB431542
SEM	standard error of the mean
SOX9	sex determining region Y-box 9
SOX17	sex determining region Y-box 17
SST	somatostatin
SSTR	somatostatin receptor
STZ	streptozotocin
T1DM	type 1 diabetes mellitus
T2DM	type 2 diabetes mellitus
TCA	tricarboxylic acid cycle
TF	transcription factor
TSH	thyroid stimulating hormone
VEGF	vascular endothelial growth factor
VIP	vasoactive intestinal peptide

Wnt3a	wingless-int 3a
wpc	human weeks post-conception

CHAPTER 1:
General Introduction

1.1. Diabetes Mellitus

Diabetes Mellitus (DM) is a metabolic disorder characterized by hyperglycemia or high blood glucose levels. Elevated blood glucose concentrations in diabetic patients are due to inadequate synthesis and secretion of insulin from the pancreas (insulin deficiency) or desensitization of insulin signaling response (insulin resistance) [1]. Insulin is a hormone that maintains glucose homeostasis: it regulates liver, skeletal muscle and adipose tissue to absorb glucose from the blood thereby reducing postprandial blood glucose levels [2]. Other symptoms of diabetes include increased urination (polyuria), increased thirst (polydipsia), increased hunger (polypagia), and weight loss [3]. Currently, diabetes is an emerging epidemic that is expected to affect more than 360 million people worldwide by 2030 [4]. The aging population of North America and rising obesity rates further exacerbate the prevalence of the disease. Importantly, diabetes is a risk factor for many vascular diseases such as stroke, limb ischemia and heart attack along with retinopathy and neuropathic disorders, which will take an enormous toll on global healthcare costs in the near future.

1.1.1. Types of Diabetes Mellitus

Type I Diabetes Mellitus (T1DM) is caused by auto-immune destruction of insulin-secreting β cells of the islets of Langerhans in the pancreas resulting in absolute insulin deficiency. Major players of this auto-immune reaction are autoantibody producing B cells [5], CD4⁺ T helper cells [6], CD8⁺ T cells [7], and monocytes and macrophages of the innate immune system [8]. Onset of T1DM is early, usually diagnosed in children and adolescents. Patients afflicted with this condition are entirely

dependent upon exogenous sources of insulin for maintaining glucose homeostasis. If untreated T1DM is fatal; however, despite insulin supplementation children diagnosed with T1DM by age 10 are estimated to have a life expectancy 18 years shorter than the non-diabetic population [9].

In contrast, Type II Diabetes Mellitus (T2DM) is the result of insufficient secretion of insulin by β cells due to insulin resistance [10]. Insulin resistance is characterized by the decreased sensitivity of cells to insulin. Insulin resistant cells fail to adequately absorb circulating insulin causing hyperglycemia. β cells respond to decreased insulin sensitivity by increased insulin secretion resulting in compensatory hyperinsulinemia but are still unable to overcome insulin resistance [10]. This condition is known as relative insulin deficiency, a hallmark of T2DM. During late stages of T2DM, β cell toxicity and exhaustion reduces insulin secretion, leading to insulin dependence [11].

Unlike T1DM patients that depend solely on exogenous insulin injections, T2DM can be managed using drug-based treatment options combined with life-style adjustments such as increased physical activity. Drugs that act to lower blood glucose levels or to enhance insulin secretion by β cells are the two primary treatment options. Metformin, one of the most commonly prescribed medications for T2DM, acts by suppressing glucose production and systemic release by the liver (hepatic gluconeogenesis), and enhances insulin sensitivity in muscle and adipose tissue, thereby lowering blood glucose [12]. Another class of anti-diabetic drug, sulfonylurea derivatives, acts to enhance insulin secretion by β cells to compensate for insulin resistance in the body [12]. Despite these treatment options, the relative risk of death for T2DM is two times higher than the non-

diabetic population, resulting in significant reduction in life-expectancy of individuals with T2DM [13].

1.2 Pancreas Biology

In mammals, the pancreas is located in the abdomen posterior to the stomach attaching to the duodenum. It is an essential organ for nutrient metabolism consisting of exocrine and endocrine components.

1.2.1 The Exocrine Pancreas

By mass the pancreas is primarily an exocrine organ; approximately 98% of the pancreas is composed of either acinar or ductal cells whose primary function is nutrient digestion. The cells are filled with secretory granules containing precursor proteases that are activated once released into the duodenum along with pancreatic lipase and amylase [14]. The pancreas responds to signals from the duodenum and secretes digestive enzymes along with bicarbonate ions to neutralize the acidic chyme released from the stomach [14].

1.2.2. The Endocrine Pancreas

Approximately 2% of pancreas mass is composed of endocrine cells organized in small highly vascularized structures called islets of Langerhans. The primary function of the islets of Langerhans is maintaining blood glucose homeostasis. There are five different types of endocrine cells within islets: α cells that secrete glucagon, β cells that secrete insulin, δ cells that secrete somatostatin, F cells (or PP cells) that secrete the

hormone pancreatic polypeptide (PP) and ϵ cells that secrete ghrelin. In mice, α cells are located in the periphery of the islets forming a cortex of cells surrounding the more numerous β cells, which are located in the islet core [15] (**Figure 1.1**). In contrast, in humans β cells do not reside at the center of the islets but are intermingled with α and δ cells [15] (**Figure 1.1**).

1.2.2.1 α cells

α cells are the second most abundant cells of the islets of Langerhans in both mice and humans [15]. Their primary function is to synthesize pro-glucagon, which is proteolytically processed and secreted as the hormone glucagon. Glucagon is a 29 amino acid peptide that regulates blood glucose concentration by promoting glycogenolysis and gluconeogenesis in the liver [16], processes that increase plasma glucose levels. Glucagon acts on cells via G protein-coupled receptors [17], which have been identified in multiple tissues including liver, kidney, intestines, pancreas and brain [18].

Glucagon is synthesized as a much larger precursor peptide called proglucagon. Upon modification in the rough endoplasmic reticulum (RER) it is processed to proglucagon and it is further modified to functional glucagon by subtilisin-like proprotein convertase 2 (PC2) [19]. The principle factor that regulates glucagon secretion is serum glucose concentration [20], with the hyperglycemic state being inhibitory and hypoglycemic state being stimulatory. Peptides such as acetylcholine [21], epinephrine [22] and norepinephrine [23] have been shown to stimulate glucagon secretion, while somatostatin and insulin secretion have an inhibitory effect on glucagon secretion [24,25].

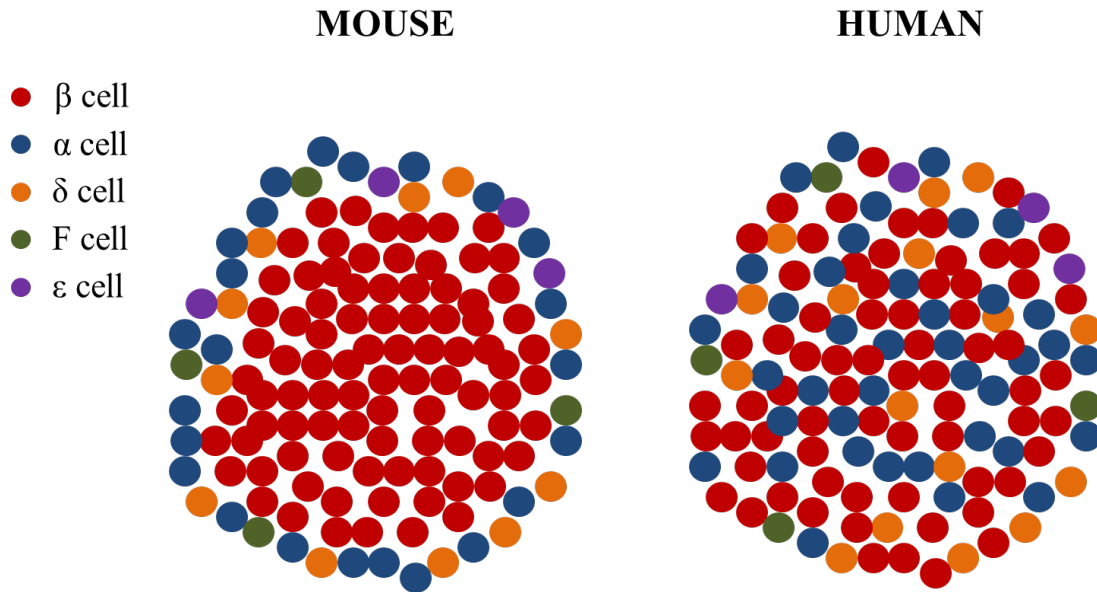


Figure 1.1. Schematic representation of cellular organization and composition of murine and human islets of Langerhans. In murine islets β cells are organized in the islet core and make up approximately 77% of all endocrine cells, while α cells make up approximately 18% of the islet and are located in the islet periphery along with other endocrine cells [15]. In contrast, human islets lack an organized core with cells intermingled with each other, and are composed of roughly 50% β cells, 35% α cells and 10% δ cells [15].

1.2.2.2 β cells

β cells are the most abundant endocrine cells of the pancreas. In mice, approximately 77% of all islet endocrine cells are β cells, while in humans islet endocrine cells are composed of 50% β cells and a greater proportion of α and δ cells than mice [15]. β cells secrete insulin, proinsulin, C-peptide and amylin into the bloodstream. The function of β cells is to maintain glucose homeostasis. β cells secrete insulin in response to postprandial blood glucose concentration, and in turn insulin enables body cells to absorb and metabolize circulating glucose to bring glucose concentrations back to basal levels [26]. The biosynthesis of insulin is highly regulated by glucose. Studies have shown that proinsulin gene transcription and biosynthesis positively correlates with plasma glucose levels [27]. This is indicative of the adaptive response to elevation of glucose in the blood.

The insulin gene is located on chromosome 11 in humans. The full insulin transcript codes for the 110 amino acid preproinsulin [28], which is cleaved in the ER to generate the 86 amino acid proinsulin [29]. Proinsulin is transported from the ER to the Golgi apparatus and subsequently into immature secretory vesicles where it is processed to insulin and C-peptide by coordinated action of proprotein convertase (PC) 2 and PC 1/3 [30]. Within the proinsulin peptide, amino acids 1 to 30 constitute the B chain of insulin, amino acids 66 to 86 constitute the A chain of insulin, and residues 31 to 65 form the cleaved C-peptide moiety [31]. The monomeric insulin consists of the A chain and B chain linked together by disulfide bridges. Thus, insulin and C-peptide are secreted in equimolar amounts by β cells. Proinsulin may be is also secreted into the blood stream and has greater half-life [32], but a much lower biological potency than insulin [33].

Although β cells respond to many other nutrients in the circulation, including amino acids and fatty acids, the extent of insulin secretion in humans is much greater in response to glucose when compared with other stimuli. In β cells, glucose metabolites trigger insulin secretion into the bloodstream. Glucose enters β cells by the glucose transporter 2 (GLUT2) and is phosphorylated by glucokinase (GCK), and initiates glycolysis and ATP production [34]. Elevation of the cytosolic ATP to ADP ratio blocks ATP-dependent K^+ channels, which in turn results in membrane depolarization and subsequent opening of voltage gated Ca^{+2} channels [35]. Increase cytosolic Ca^{+2} concentrations represents the main trigger initiating the exocytosis of insulin, proinsulin and c-peptide containing vesicles [35]. This process is termed glucose-mediated insulin secretion (**Figure 1.2**).

1.2.2.3 δ cells

In humans, δ cells comprise roughly 8-12% of all islet endocrine cells [15] and are responsible for the synthesis and secretion of the hormone somatostatin. Apart from the pancreas, somatostatin is also secreted by δ cells in the stomach and neuroendocrine cells in the periventricular nucleus of the hypothalamus. Functional forms of somatostatin exist as a 28 amino acid peptide or a 14 amino acid peptide. Somatostatin-14 is the C-terminal portion of somatostatin-28, and represents the primary isoform responsible for its physiological function [36]. δ cells predominately secrete the 14 amino acid form, while intestinal mucosal cells secrete the 28 amino acid form [37].

Somatostatin secretion is regulated by glucose [38], glucagon-like peptide 1 (GLP-1) [39], and gastrin [40]. δ cells express Ryanodine Receptor 3 (RyR3), a Ca^{+2} -

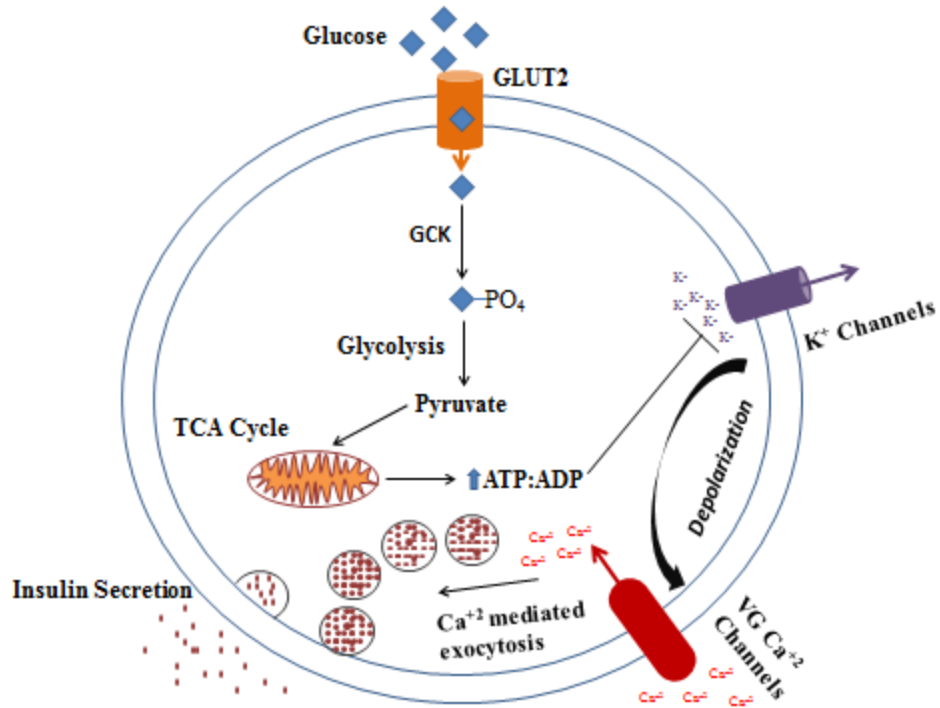


Figure 1.2. Schematic representation of glucose-mediated insulin secretion in β cells. Glucose enters β cells via GLUT2, and upon its metabolism the cytosolic ATP:ADP ratio increases resulting in inhibition of K^+ channels. Increased cytosolic concentration of K^+ results in plasma membrane depolarization and subsequent activation of voltage-gated Ca^{+2} channels. Cellular Ca^{+2} influx initiates the exocytosis of insulin containing vesicles.

channel shown to be responsible for glucose-mediated somatostatin secretion via Ca^{+2} -induced Ca^{+2} release mechanism. Glucose metabolites induce Ca^{+2} secretion from internal ER stores, and the resulting increase of cytosolic Ca^{+2} concentration leads to activation of RyR3 Ca^{+2} channels further increasing the cytosolic Ca^{+2} concentration, which activates exocytosis of somatostatin secretory vesicles [38]. δ cells also express Cholecystokinin (CCK) B Receptor (CCK_{BR}), which upon binding with CCK and Gastrin promotes somatostatin secretion [40]. Somatostatin inhibits the secretion of several hormones such as growth hormone (GH) [41] and thyroid stimulating hormone (TSH) [42] from the anterior pituitary, pancreatic hormones [43], and gastro intestinal peptide hormones such as vasoactive intestinal peptide (VIP) and gastrin [44]. Somatostatin receptors (SSTRs) coupled to $\text{G}_{i/o}$ proteins have been identified in gut endocrine G cells [45], and pancreatic endocrine α and β cells [46]. Activation of SSTRs inhibits hormonal secretion by suppressing cAMP and Ca^{+2} influx via voltage-gated Ca^{+2} channels, thereby directly reducing exocytosis [47]. Importantly, somatostatin activity is known to decrease glucose-stimulated insulin secretion in β cells.

1.2.2.4 F cells

F cells (or PP cells) constitute a very small portion of the human islets of Langerhans [15]. Their primary function is the synthesis and secretion of PP, a hormone known to regulate gastrointestinal motility and secretions. PP acts to reduce the rate of gastric emptying [48], gall bladder emptying [49], and pancreatic exocrine secretion [50]. PP secretion is promoted by insulin-induced hypoglycemia and direct vagal nerve stimulation [51]. Hyperglycemia inhibits PP secretion [52].

1.2.2.5 ϵ cells

ϵ cells synthesize and secrete ghrelin, a hormone which is also produced by D1 cells residing in the fundus of the stomach. Ghrelin is a 28 amino acid peptide that acts as a hunger-inducing hormone by activating the mesolimbic cholinergic-dopaminergic circuitry that reinforces rewards such as food [53,54]. Therefore, it is suggested that ghrelin antagonists could be a potential treatment for obesity. Furthermore, ghrelin has been shown promote the release of GH via activation of the G-protein coupled receptor GSH-R1a in the anterior pituitary [55]. Ghrelin secretion is primarily regulated by food intake.

1.3. Pancreatic Development

During the blastula stage of development, the embryo is composed of a single layer of epiblast cells which become organized into a three-layered structure during gastrulation. Gastrulation occurs when epiblast cells around the newly formed primitive streak undergo epiblast to mesenchymal transition and ingress through the primitive streak giving rise to three germ layers [56]. Gastrulation is followed by organogenesis, where each of the three germ layers, the ectoderm, mesoderm and endoderm, gives rise to specific tissues and organs. The ectoderm gives rise to epidermis and cells of the nervous system [57], the mesoderm gives rise to the notochord, muscle, bone, cartilage and other connective tissues [58-60], and the endoderm gives rise to the respiratory system and organs associated with the digestive system such as the pancreas [61].

1.3.1. Formation of the Pancreas from Definitive Endoderm.

Prior to organogenesis, the endoderm undergoes extensive patterning along the anterior-posterior axis. The definitive endoderm gives rise to the primitive gut tube, which becomes specified into distinct organ domains along the dorsal-ventral and anterior-posterior axes into foregut, midgut and hindgut in response to signaling events initiated by the surrounding mesoderm [61]. Factors secreted by mesodermal tissues such as the notochord including Activin and FGF2 allow for pancreas and liver fate determination from the gut endoderm [61]. Both exocrine and endocrine components of the pancreas arise from a common precursor population residing in the primitive gut [62]. In mice, the first morphological signs of pancreas formation occur at embryonic day (E) 9.0, when the pancreas emerges as two epithelial buds from opposite ends of the foregut endoderm [63]. These buds contain multipotent pancreatic progenitor cells (MPCs) that will ultimately give rise to all three pancreatic lineages [64]. Lineage-tracing experiments have shown that MPCs retain the potential to develop all pancreatic lineages until E12.5. Between E9.0 to E12.5 the MPCs residing in the ventral and dorsal pancreatic epithelia proliferate and expand the progenitor population [65]. Both pancreatic buds also undergo expansion during this period and invade the surrounding mesenchyme, forming a multi-layered stratified epithelium. Between E11.5 and 12.5 the two buds merge together during rotation of the gut [66]. At E12.5 the pancreas undergoes branching morphogenesis forming tubular structures. At this stage the pancreas consists of two cell types: the epithelial tips contain MPCs which eventually give rise to acinar progenitor cells by E13.5, while the tubular or trunk regions are comprised of cells that will form either ducts or endocrine cell types [66].

Differentiation of endocrine pancreas cells is first seen in the dorsal bud, where glucagon-positive cells emerge around E9.5, and insulin positive cells emerge around E10.5 in both buds [67]. E12.5 to E15.5 is a period in which pancreas progenitors undergo extensive proliferation and terminal differentiation into endocrine or acinar cell fates [66]. Also during this phase of endocrine differentiation, the pancreatic endocrine cells begin to organize into cell clusters, which eventually coalesce post-natally to form islets.

Similar to mice, in humans, the pancreas forms from the fusion of dorsal and ventral pre-pancreatic buds of the primitive gut. The dorsal pancreatic bud is first evident morphologically at day 26 post-conception (dpc) and the fusion of the buds occurs by 56 dpc [68]. Endocrine differentiation appears to begin around 8 weeks post-conception (wpc). Furthermore, human endocrine cells start coalescing into islet-like cell clusters around 11-14 wpc [68], and undergo a final reorganization after 21 wpc with intermingled β and α cells [69].

1.3.2. Role of Transcription Factors in Pancreatic Development

Transcription factors (TFs) are crucial components of gene regulatory network; they interact with each other as well as other proteins to control mRNA and consequentially, protein expression. During development TFs control the expression of genes necessary for cell fate specification and cellular differentiation. The formation of the pancreas depends on specific signaling events, which regulate TF activity. Cell type-specific regulatory networks, consisting of interactions between TFs and extrinsic signals,

guide the pancreatic progenitor population in a context-dependent manner to their programmed fate.

1.3.2.1 SOX17

Sex Determining Region Y (SRY) – High Mobility Group Box (HMG-box) 17 (SOX17) is part of a family of transcription factors involved in regulation of embryonic development and cell fate determination. SOX17 belongs to the Sox-subgroup F along with SOX7 and SOX18. The HMG-box is the DNA-binding domain composed of three alpha helices separated by loops, a highly conserved domain among eukaryotic species [70].

SOX17 is essential for the development of definitive endoderm: in *Sox17* knockout mice the definitive endoderm is depleted and the embryonic gut and all its associated organs fail to form [71]. Sox17 binds to the minor groove of DNA at the ATTGT consensus motif and regulates the transcription of genes involved in definitive endoderm specification during gastrulation [72]. The C-terminus of Sox17 has been shown to bind to β -catenin, and this is important for transcription of several endodermal genes including Hepatocyte Nuclear Factor (HNF) 1 β , Forkhead box protein (FOX) A1 and FOXA2. [73].

1.3.2.2 Early Pancreatic Transcription Factors (HNF1 β , HNF6, PDX1, SOX9 and PTF1a)

The earliest transcription factors that mark the MPCs residing in the ventral and dorsal pancreatic buds are HNF1 β , HNF6, Pancreatic and Duodenal Homeobox 1

(PDX1), SOX9 and Pancreas-specific Transcription Factor 1a (PTF1a) [66]. During mouse development, *Hnf1 β* is expressed in the foregut endoderm at E8 prior to the formation of pancreatic endoderm [74], and at later stages of pancreatic development its expression is maintained exclusively in duct cells [75]. *Hnf1 β* regulates the expression of *Hnf6*, which is an important regulator of *Pdx1* expression, a key early pancreatic progenitor marker [76]. It has been suggested that sequential activation of *Hnf1 β* , *Hnf6* and *Pdx1* gives rise to pancreatic multipotent progenitor populations [76], whereas *Sox9* is implicated with MPC proliferation [77].

Lineage tracing experiments in mice have demonstrated that *Pdx1* expressing multipotent cells give rise to all pancreatic cell types [78]. *Pdx1* marks the pre-pancreatic tissue of the endoderm since its expression is initiated at E8.5, prior to the morphological changes associated with the development of the ventral and dorsal pancreatic buds [79]. *Pdx1* is essential for early pancreatic development and precursor cell expansion. *Pdx1*-null mice showed impaired pancreatic development by E10.5 and lack a pancreas at birth [80]. At later stages of pancreatic development *Pdx1* is expressed in β cells [81]. Apart from the pancreas, *Pdx1* is expressed in the other endoderm-derived endocrine cells such as gastric enteroendocrine cells of the developing gut [82,83]. For example, G cells are absent in *Pdx1*-null mice, suggesting that *Pdx1* is important for the development of gastrin-secreting cells [83].

Similar to *Pdx1*, pancreas-specific *Sox9* knock-down results in loss of MPC proliferation [78]. *Sox9* has also been implicated in the maintenance of MPC identity through direct regulation of key MPC genes including *Hnf1 β* , *FoxA2* and *Hnf6* [84]. Another TF crucial for the commitment and expansion of MPCs is PTF1a. *Ptf1a*-

deficient cells do not remain committed to the pancreatic fate and adopt an intestinal fate *in vivo* [85]. Furthermore, *Pdx1* and *Ptf1a* have the ability to guide duodenal precursors to pancreatic fate when expressed ectopically [85].

1.3.2.3 NGN3

In early pancreatic development, MPCs have the potential to give rise to all three pancreatic cell types including endocrine cells. Neurogenin 3 (NGN3), a basic helix loop helix (bHLH) TF, is necessary for endocrine specification of MPCs. NGN3-positive precursor cells give rise to all five pancreatic endocrine cell types [62]. *Ngn3*-null mice are devoid of pancreatic endocrine cells but demonstrate normal acinar and ductal development [86]. NGN3 directly induces the expression of other TFs that are important for pancreatic endocrine differentiation, including paired-box containing gene (PAX) 4, Insulinoma-associated 1 (INSM1) and regulatory factor X (RFX) 6 [87]. Expression of *Ngn3* occurs in a biphasic manner in the developing mouse pancreas: *Ngn3* expression first occurs at E9.5 and is downregulated by E10.5 and then *Ngn3* expression peaks again around E12.5 and diminishes permanently from pancreatic endocrine progenitors by E18 [88]. In contrast, the human embryonic pancreas demonstrates a single phase of NGN3 expression which is maintained from 8 wpc up to 21 wpc [89].

1.3.2.4 Additional Pancreatic Endocrine Transcriptional Factors

Following NGN3 activation, several downstream TFs are activated that guide pancreatic endocrine progenitors to one of the five cell fates. *Insm1* is expressed early in the endocrine specification pathway and *Pax4* is expressed shortly after and has a more

specific role. This is supported by studies showing that *Insm1*-deficient mice demonstrate a deficiency in all endocrine cell types [90], whereas *Pax4*-null mice only show diminished numbers of β and δ cells, along with increased numbers of α and ϵ cells [91]. Thus, *Pax4* serves to specify NGN3-positive progenitors to the β and δ cell fates, while repressing the α and ϵ cell fates.

Another important TF that directs *Ngn3*-positive endocrine specifications is *Arx*. *Ngn3*-positive progenitors express both *Pax4* and *Arx*, but *Pax4* expression restricts endocrine progenitors to β and δ cell fates while *Arx* expression restricts them to α cell fate [91]. This is supported by studies showing *Arx*-null mice having the opposite phenotype of *Pax4*-null mice: complete loss of α cells but an increase in both β and δ cell types [92]. In addition to enhancing the expression of certain TFs, repression of specific TFs is required to maintain the phenotype of mature endocrine cells. For example, the continual repression of *Arx* expression in β cells is important for the maintenance of β cell phenotype and identity. The repression of *Arx* expression is maintained by *Nkx2.2* and *Nkx6.1* [93,94].

Following *Pax4* expression, *Pax6* plays an important role for further maturation of α , β and δ cells. *Pax6*-null mice show a complete loss of α cells, and diminished numbers of β and δ cells, and an increase in ghrelin-positive cells [95]. *Pax6* expression is maintained in mature α , β and δ cells and it serves to regulate insulin, glucagon and somatostatin gene transcription [95].

Another late stage endocrine marker is NKX6.1, the expression of which is downstream of NKX2.2. Studies investigating human endocrine progenitor cells have shown that NKX6.1-positive precursors give rise to insulin-positive and somatostatin-

positive cells, while NKX6.1-negative precursors give rise to greater proportions of glucagon-positive cells and lower proportion of β and δ cells [96]. This suggests that NKX6.1-positive progenitors have the ability to adapt either β or δ cell fates at the expense of the α cell fate. However, upon maturation NKX6.1 expression is maintained exclusively in β cells [97]. *Nkx2.2* has been shown in mice to be necessary for the promotion of terminal β cell differentiation [98] and suppression of δ cell phenotype by repressing somatostatin gene expression [99] (**Figure 1.3**).

β and δ cells have common regulators during development, and also show common patterns of TF expression in mature cell types (**Figure 1.3**). For instance, PDX1 expression is restricted to mature β cells and a subpopulation of δ cells. In rat, Pdx1 and Pax6 have been shown to bind to the upstream somatostatin enhancer to promote gene transcription [100]. In β cells PDX1 directly binds to the insulin gene promoter resulting in activation of gene expression [101]. Another mature endocrine TF, insulin gene enhancer protein 1 (ISL1) enhances transcription of both insulin and somatostatin by binding to insulin enhancer elements and the somatostatin promoter, respectively [102].

MafA and *Nkx6.1* are considered hallmarks of the mature β cell phenotype since they are uniquely expressed in β cells. These genes are also crucial for mature β cell function. *MafA*-null mice show reduced transcription of *insulin*, *Pdx1* and Glucose transporter type 2 (*Glut2*) [94]. Similarly, *Nkx6.1* directly controls the expression of genes that maintain the β cells function, including *Glut2*, the proinsulin-to-insulin convertase enzyme *PCI*, proteins associated with insulin protein folding and maturation such as endoplasmic reticulum oxidoreductin 1- β (*ERO1- β*), and the islet-specific zinc

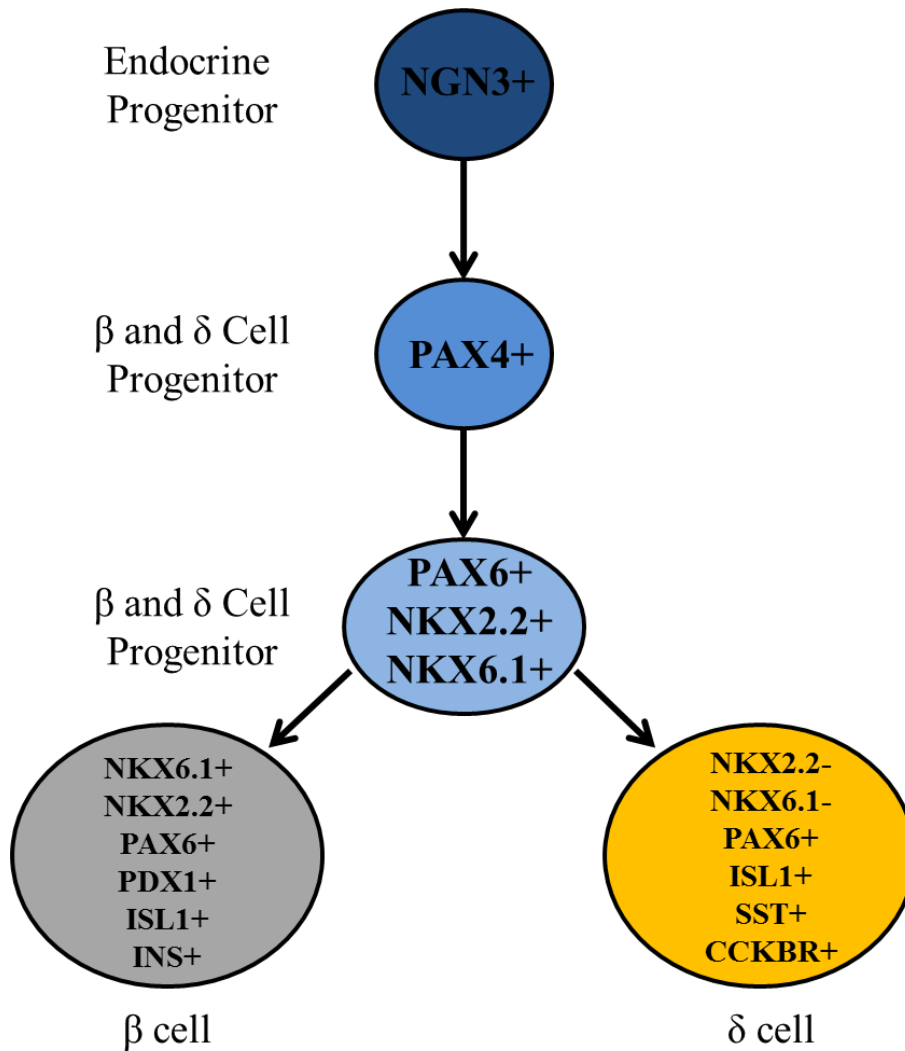


Figure 1.3. Summary of transcription factor-mediated pancreatic endocrine specification during mammalian development. NGN3 activates the transcription of PAX4, which guides progenitor cells towards β and δ cell fates at the expense of other endocrine cell fates. Downstream of PAX4, PAX6 also promotes β and δ cell phenotype and its expression is maintained in both mature cell types. Whereas, NKX2.2 suppresses δ cell specification and promotes terminal β cell differentiation. Consequentially, mature δ cells do not express NKX2.2 and NKX6.1.

transporter (*ZnT-8*) important for insulin secretion [97]. *Nkx6.1*-null mice show impaired insulin biosynthesis and insulin secretion, and a rapid onset of hyperglycemia and hypoinsulinemia [97].

1.4. Stem Cells

Stem cells are primitive cells with the ability to differentiate into a mature specialized cell types or self-renew and maintain their primitive phenotype [103]. There are two main categories of stem cells: pluripotent stem cells, and adult stem cells. Embryonic stem cells and induced pluripotent stem cells (iPSC - first derived from murine fibroblast by ectopic expression of the pluripotency associated factors Oct4, Sox2, c-Myc, and Klf4 [104]) have the ability to generate cells corresponding to all three embryonic germ layers. In contrast, adult stem cells typically reside within postnatal tissues and demonstrate a comparatively restricted differentiation potential, and act primarily to repair and replenish adult tissues [105].

1.4.1. Human Embryonic Stem Cells (hESC)

The first human embryonic stem cells (hESC) were derived from blastocysts in 1998 by James Thompson [106]. hESCs can generate mesoderm, endoderm and ectoderm-derived cell types following spontaneous embryoid body-induced differentiation *in vitro* and teratoma formation *in vivo*, thus demonstrating their pluripotent potential [107]. To permit stem cell experimentation, optimal culture conditions have been developed enabling hESC cell lines to be maintained *in vitro* indefinitely without loss of self-renewal or differentiation potential [108-110]. Due to

their unlimited potential to differentiate into all mature human cells types, hESC represent an exciting tool for future cell therapy applications.

1.4.2. Human Bone-Marrow derived Multipotent Stromal Cells

Multipotent stromal cells (MSCs) were first derived from the bone marrow of adult organisms[111]. MSCs, also known as mesenchymal stem cells, are fibroblast-like cells that grow adherent to plastic and can differentiate into osteoblasts, adipocytes and chondrocytes [112]. In addition to residing in the bone-marrow compartment [113], MSCs have been localized to the connective tissues of most organs including skeletal muscle [114], adipose tissue[115], lung [116], kidney [117], pancreas [118] and the umbilical cord [119]. It has been demonstrated that MSCs are present in adult organs throughout the body in close association with pericytes [118]. The perivascular association of MSCs in adult tissues may be due to their physiological role of localizing to sites of tissue injury and inducing tissue regeneration and repair. MSCs have been shown to enhance endogenous repair in various animal models such as lung disease [120], kidney disease [117], diabetes [121], and graft versus host disease with minimal signs of long-term engraftment following injection *in vivo* [122]. Despite low tissue engraftment, the regenerative effects of MSCs suggests that they regulate tissue repair by creating a regenerative microenvironment through the secretion of soluble paracrine factors rather than differentiating to replace cells within damaged tissue [123].

Progenitor subpopulations of MSCs can be purified from the non-hematopoietic component of the human BM based on high aldehyde-dehydrogenase (ALDH) activity,

an enzymatic function conserved in endothelial, hematopoietic and mesenchymal progenitors [121,124]. ALDH high cells have been shown to have higher regenerative potential than unsorted BM cell populations [121,125,126].

1.5. Cell-Based Therapies for Diabetes Mellitus

1.5.1. Islet Transplantation

Islet-transplantation therapy, commonly known as the Edmonton Protocol, was developed using islets isolated from cadaveric donor pancreas [127]. These isolated islets were transplanted into severely type 1 diabetic patients via the hepatic portal vein and immunosuppressants were administered to protect the newly transplanted cells. All seven patients transplanted became insulin-independent in the first year of transplantation [127]. This procedure provided the proof-of-principle that insulin independence can be achieved using cell-based therapeutics for the treatment of diabetes. However, the severe shortage of cadaveric donors paired with the requirement of islets from approximately two to three donors per transplant precludes the widespread use of such therapy.

1.5.2. Differentiation of Embryonic Stem Cells to Islet Cells.

Due to the limited supply of cadaveric islets it has become a priority to generate alternative sources of insulin-producing cells for transplantation. Pluripotent cells including hESC and iPSC have the potential to provide an unlimited source for cell-based regenerative therapies. Given the unlimited potential and proliferative capacity of hESC, efforts have been made to drive their differentiation towards the β cell fate by attempting to recapitulate signals present during embryonic development [128-131].

1.5.2.1 Growth Factor Based Differentiation

Based on growth factor signaling during embryonic development, step-wise protocols have been developed to produce islet cell populations from pluripotent human cells, including glucose-responsive β cells [129,131]. During gastrulation, Wnt and TGF- β secretion from the primitive streak is essential for specification of the definitive endoderm [132,133]. In particular, signaling events triggered by Nodal, a member of TGF- β family, are critical for definitive endoderm specification [134,135]. The first reports of definitive endoderm differentiation from hESC [136] used the TGF- β member Activin A as a substitute for Nodal, since it is readily available and binds to the same receptors as Nodal [137]. Expression of the definitive endoderm genes CXCR4, SOX17 and FOXA2 were enhanced after 5 day treatment with Activin A. To generate insulin-positive β cells, a landmark study developed a stepwise protocol in which definitive endoderm cells were subsequently treated with FGF10 for 3 days to generate equivalent of primitive gut tube, followed by noggin, KAAB-cyclopamine and retinoic acid for 3 days to generate PDX1-positive pancreatic progenitors, and finally cultured in DMEM supplemented with B27 until 20 days post-definitive endoderm differentiation when C-peptide levels were detected [131]. Although these studies established that recapitulation of embryonic growth factor signaling can be used to guide hESC differentiation, the clinical application of these findings was limited by low efficiency of c-peptide positive cell differentiation (7%) that were not able to respond to glucose stimulation and the generation of polyhormonal cells [129].

Building from these initial studies, numerous protocols for β -cell generation have been developed using various combinations of growth factors, mitogens and small

molecules that induce pleiotropic effects via activation of multiple, interacting signaling pathways. Although these protocols induce pancreatic endocrine differentiation from hESC and accomplish in 25 days what would take 21 weeks in humans, they are limited as they produce heterogenous populations containing polyhormonal cells, with severe restriction of cell proliferation from the onset of differentiation [129,131]. Furthermore, these protocols do not achieve functional maturation of endocrine cells *in vitro*, requiring up to 3 months transplantation *in vivo* to detect glucose-mediated insulin secretion [130]. Although recent protocols have demonstrated the ability to generate monohormonal insulin-positive cells that exhibit glucose responsiveness after differentiation *in vitro*, they are only 20% as efficient at secreting insulin as adult islet β cells [138]. Thus, despite recent advances, protocols are still unable to recapitulate appropriate cell maturation, yielding cells unable to adequately secrete insulin in response to elevated blood glucose.

1.5.2.2 Transcription Factor Based Differentiation

Previous studies have established the importance of endoderm specification of hES cells to achieve efficient pancreatic endocrine differentiation [129]. Attempts to differentiate pancreatic endocrine cells from embryonic stem cells without endoderm specification led to the development insulin-expressing neural cell precursors [139,140]. Transcription factor-based protocols represent an alternate strategy to generate pancreatic cell types from hESCs that may lead to more efficient outcomes.

The use of transcription factor-based strategies for pancreatic differentiation has been recorded extensively in literature. For instance, ectopic expression of Pdx1, Ngn3

and Mafa to reprogram pancreatic exocrine cells to β cells has been previously established [141]. Other strategies involve ectopic *Ptf1a* expression. *Ptf1a* expression in *Xenopus* endoderm induced the formation of endocrine pancreatic tissue but also led in the generation of exocrine fate [142]. Similarly, ectopic overexpression of *Ptf1a* in murine ES cells promoted the generation of exocrine, ductal and endocrine cell fates [143]. Previous research has demonstrated that transcription factor-mediated differentiation can generate proliferative, homogeneous, and lineage-restricted definitive endoderm progenitors (DEPs), that are receptive to signals for further cell-type-specific maturation. Definitive endoderm specification from hESC was achieved by ectopic expression of the transcription factor SOX17 in pluripotent stem cells [144]. Building on the concept of transcription factor-based differentiation, recent studies by the Séguin lab assayed for the ability of islet-specific transcription factors (PDX1, NGN3, PAX4) to specify pancreatic endocrine cells from definitive endoderm progenitors (*Sattin and Watts, manuscript in preparation*). These studies demonstrated that ectopic expression of either PAX4 or PDX1 was not sufficient to specify pancreatic cell types from hESC or DEPs. In contrast, sequential activation of SOX17 and NGN3 in hESC cells resulted in cells that express hallmarks of pancreatic β cell precursors (PAX4, PDX1, NKX6.1, NKX2.2, INSULIN). Importantly, NGN3 expression in the absence of SOX17-mediated DE specification did not promote this phenotype. Similarly, other studies have shown that ectopic expression of Ngn3 in *Xenopus laevis* endoderm promotes β and δ cell development [145]. Collectively, these data provide the proof-of-principle that NGN3 expression can direct definitive endoderm cells towards the pancreas endocrine lineage.

1.5.3 Islet Regeneration Mediated by Human MSCs

A second strategy to restore islet function is the use of adult stem cells to stimulate endogenous new islet formation to restore the β -cell mass. Transplantation of human bone-marrow (BM) derived stem cells has been shown to induce islet-regeneration and reverse hyperglycemia in streptozotocin (STZ)-treated non-obese diabetic severely compromised immune-deficient (NOD/SCID) mice [146]. Following MSC transplantation, STZ-treated NOD/SCID mice showed an increase in serum insulin levels and increased β cell mass [121]. Transplanted MSCs showed engraftment in the pancreas *in vivo* but did not exhibit endocrine characteristics, suggesting the rescue of hyperglycemia in STZ-treated mice was due to the improved function of recipient islets and endocrine cell proliferation [146]. It was subsequently shown that MSCs induced the formation of small islet clusters, suggesting the induction of new islet formation in STZ-treated mice [147].

Although the precise mechanism underlying endogenous islet regeneration is unknown, it has been suggested that MSCs release factors and cytokines that induce adult pancreatic endocrine progenitors to form new islets [121]. This idea is supported by lineage-tracing experiments that demonstrate that the adult pancreas contains multipotent progenitors that can give rise to mature endocrine cells [148]. Recent studies have demonstrated that adult BM-derived MSCs induce islet neogenesis within CK19-positive pancreatic ductal regions leading to CK19-positive cells found within new islets [121,147]. Collectively, these studies suggest that MSCs guide endocrine progenitor populations residing in the ductal regions of adult pancreas to differentiate into mature islets.

The islet-regenerative function of MSCs has been shown to vary considerably from donor to donor, leading to the functional classification of MSC samples as regenerative or non-regenerative based on their ability to induce islet neogenesis following in vivo transplantation [126]. Transcriptome analysis of these subset has established their distinct signatures: regenerative MSCs demonstrate increased expression of insulin-like growth factor binding protein 5 (IGFBP5), downstream effectors of Wnt-signaling such as Wnt1-inducible-signaling pathway protein 1 (WISP1) and secreted frizzled-related protein 1 (SFRP1), epidermal growth factor receptor (EGFR) ligands such as amphiregullin (AREG), modulators of the transforming growth factor beta (TGF- β) and also showed increased secretion of pro-angiogenic proteins and show decreased secretion of anti-inflammatory cytokines than non-regenerative MSCs [121]. These signaling pathways and their effectors are implicated as potential regulators of MSC-induced islet regeneration [121]. Interestingly, several of these candidate effectors are essential components of protocols developed to differentiate pluripotent stem cells to pancreatic endocrine cells, including TGF- β /activin signaling [129,131]. Furthermore, EGF treatment has been shown to promote islet function and reverse hyperglycemia after onset of diabetes in NOD mice [149]. Furthermore, EGFR signaling has been shown to induce β cell mass expansion in mice [150]. The increased expression and secretion of factors implicated with enhanced islet-function by islet-regenerative MSCs highlights the importance of paracrine signaling in MSCs-induced islet regeneration.

1.6. Strategies for Transcription Factor Based Differentiation of hESC

1.6.1. SOX-17 ER^{T2} System

Previous studies in Séguin lab used constitutive transcription factor overexpression models to study stem cell differentiation, which differ from the inducible transgene expression strategy undertaken by the current study. Building on SOX17-mediated differentiation of hESC to DEPs, it was necessary to refine our strategy to more accurately recapitulate the developmental pattern of transcription factor activation by generating inducible cell lines that allow to down-regulation of SOX17 following DEP specification. hESC lines with inducible SOX17 expression (CA2-pCAGG-SOX17-ER^{T2}) were previously generated in the Séguin lab. SOX17-ER^{T2} is a fusion protein of SOX17 and a mutant form of human estrogen receptor (ER^{T2}) that does not bind to its endogenous ligand (17 β -estradiol) at physiological concentrations but will bind to 4-hydroxytamoxifen (4OHT) at low concentrations, promoting ER^{T2} nuclear localization.. In these cells, SOX17-ER^{T2} is constitutively expressed under the control of strong cytomegalovirus (CMV) early enhancer/chicken β actin promoter (CAGG), and upon treatment of cells with 4OHT it becomes localized to the nucleus allowing SOX17 to function as a transcription factor.

1.6.2. NGN3 piggyBac System

In order to control differentiation downstream of SOX17 activation, we made use of a second gene expression system that would confer inducible expression of NGN3. We employed the transposon-based Tet-inducible piggyBac system [151] for NGN3 (PB-NGN3) which is comprised of three separate vectors: 1) TetO-NGN3-IRES- β GEO, 2)

pCAGG-rtTA, and 3) CMV-transposase element. While the first two vectors become stably integrated into the genome, the vector expressing the transposase element is transiently expressed and enables the incorporation of the other two vectors. The PB-NGN3 system utilizes the Tet-on system of transgene induction in which reverse tetracycline transcriptional activator (rtTA) is constitutively expressed and upon binding to doxycycline binds to the operator region upstream of NGN3-IRES- β GEO. Thus, expression of NGN3 is induced following treatment of cells with doxycycline.

1.6.3. Strategies for Enhancing Maturation of Endocrine Precursors Cells

Our group has previously shown that human MSC induce endogenous islet regeneration when injected into immune deficient mice following streptozotocin-mediated β -cell destruction [121]. Although the precise mechanisms responsible for the induction of islet regeneration remain a topic under intense investigation, it has been established that MSCs promote islet regeneration *in vivo* with minimal long-term tissue engraftment, suggesting the role of MSC-secreted paracrine factors in promoting islet regeneration from resident endocrine progenitors [121]. We therefore extrapolated from these findings and developed *in vitro* strategies using MSC-conditioned media (MSC-CM) to generate a microenvironment to promote pancreatic endocrine maturation of hESC-derived precursor cells.

1.7. Rationale

Definitive endoderm specification from hESC has been achieved by ectopic expression of the transcription factor SOX17 [144]. Furthermore, preliminary studies

show that sequential expression of SOX17 and NGN3 in hESCs (using a genetic strategy that conferred constitutive transgene expression) results in cells that express hallmarks of pancreatic β -cell precursors. Importantly, pancreatic differentiation following ectopic NGN3 expression was dependent on SOX17-mediated definitive endoderm differentiation. We therefore sought to use the SOX17-ER^{T2} and PB-NGN3 transgenic cell lines to develop a protocol to create pancreatic endocrine precursor populations from hESC. Previous studies demonstrated that the generation of functional β cells from hESCs is enhanced after transplantation *in vivo* [130], demonstrating the importance of the cell microenvironment on differentiation, development and maturation. We predict that following NGN3-mediated endocrine specification of hESC, maturation will be required to promote the transition from endocrine precursors to functional and mature endocrine cells *in vitro*. Because the microenvironmental factors that control the final stages of endocrine cell maturation are largely unknown, we propose that soluble factors secreted by islet-regenerative human BM-derived MSCs will accelerate endocrine cell maturation.

1.8. Hypothesis

Hypothesis I: NGN3-mediated differentiation of SOX17-definitive endoderm will produce pancreatic endocrine precursor cells.

Hypothesis II: SOX17/NGN3 differentiated cells will be receptive to maturation signals secreted by islet-regenerative MSCs facilitating the production of mature pancreatic endocrine cell.

1.9. Objectives

Objective I: Develop a transcription factor-based protocol to differentiate hESC to differentiate pancreatic endocrine-cell precursors via sequential activation of SOX17 (definitive endoderm specification) and NGN3 (endocrine precursor specification).

Objective II: Evaluate the effects of islet-regenerative human MSC-conditioned media in directing the maturation of endocrine precursors *in vitro*.

CHAPTER 2:
Materials and Methods

2.1. Generation of NGN3 expression constructs

The open reading frame (ORF) corresponding to the human NGN3 gene was amplified by PCR using primers designed to incorporate the endogenous Kozac sequence and to add *attB* recombination sites to permit Gateway® cloning (Life Technologies). An entry clone was generated by a recombination reaction between the pDONRTM221 vector (Life Technologies) and the *attB*-NGN3 PCR product. The NGN3 ORF was introduced into the piggyBac (PB) transposon system, which allows for efficient transgene delivery in hESC [151] by Gateway® cloning. The resultant Tet-inducible piggyBac system [151] for NGN3 expression (PB-NGN3) was comprised of three separate vectors: 1) TetO-NGN3-IRES-βGEO, 2) CAGG-rtTA, and 3) CMV-transposase element. The first two vectors become stably integrated into the genome because the vector expressing the transposase element is transiently expressed and enables the incorporation of the other two vectors. The PB-NGN3 transgene utilizes the Tet-on system in which reverse tetracycline transcriptional activator (rtTA) is constitutively expressed and upon binding to doxycycline is activated and binds to the operator region upstream of NGN3-IRES-βGEO. Following cloning, the PB-NGN3-IRES-βGEO construct was verified by sequencing (**Figure 2.1**).

2.2. Generation of transgenic SOX17-ER^{T2}; PB-NGN3 inducible cell lines

hESC lines with inducible SOX17 activity (CA2-SOX17-ER^{T2}) were previously generated by the Séguin lab. In order to generate a transgenic cell line with inducible expression of both SOX17 and NGN3, the PB-NGN3-IRES-βGEO (PB-NGN3) construct was introduced along with the PB-rtTA and PB-transposase constructs into CA2-SOX17-

Figure 2.1. Alignment of the human NGN3 coding sequence with that of the PB-NGN3 transgene. Missing base pairs (N) in sequencing results were verified by aligning the unknowns to their corresponding chromatograph.

ER^{T2} cells by electroporation (170 V, 1050 μ F). rtTA integration was selected for by blasticidin resistance (5 μ g/ml) and clonal cell populations were expanded from single colonies. PB-NGN3 plasmid integration was selected for by treating replica well with doxycycline (24h; 0.5 μ g/mL) to induce PB-Tet transcription which confers neomycin resistance through the expression of beta-galactosidase neomycin fusion protein (β GEO), and transgene-positive cells were selected by G418 resistance (50 μ g/mL). Undifferentiated cells of the corresponding clonal populations in replica wells were then expanded and cryopreserved.

2.3. Cell culture

All hESC, including parental CA2, SOX17-ER^{T2}, and SOX17-ER^{T2}; PB-NGN3 were maintained using established protocols [106]. Cells were grown on γ -irradiated mouse embryonic fibroblast feeders (MEFs; SickKids ESC Facility) in KnockOut Dulbecco's Modified Eagle Medium (KO-DMEM; Life Technologies) supplemented with 15% KnockOut serum replacement (Life Technologies), 2 mM GlutaMAX, 0.1 mM nonessential amino acids (NEAA), 0.5 mM 2-mercaptoethanol, and 10 ng/mL FGF2 (all from Life Technologies). Transgenic cell populations were maintained in media supplemented with Puromycin to select for the SOX17-ER^{T2} transgene (Bioshop, 0.8 μ g/mL) or G418 (50 μ g/ml) to select for the PB-NGN3 transgene. For activation of SOX17-ER^{T2}, 4-hydroxytamoxifen (4OHT) was administered (0.2 μ M). For activation of PB-NGN3, doxycycline (DOX) was administered (0.5 μ g/mL). When cultures had attained 75% confluence, cells were passaged by enzymatic dissociation (0.05% Trypsin-EDTA; Life Technologies) at split ratio of 1:12 (every 4-6 days). All protocols were

reviewed and approved by the CIHR Stem Cell Oversight Committee (SCOC) as well as the Research Ethics Board (REB) at the University of Western Ontario.

Human BM-derived MSCs were cultured in serum-free MSC media (STEMPRO® MSC SFM; Life Technologies), supplemented with STEMPRO® MSC SFM Supplement CTS™ (Life Technologies) and 2mM L-glutamine. When cultures attained 75% confluence, cells were passaged by enzymatic dissociation (0.5% Trypsin-EDTA; Life Technologies) and seeded at a density of 10000 cells/cm².

Human ductal epithelial carcinoma cells (PANC-1) [152] were maintained in DMEM + 10% Fetal Bovine Serum (FBS). PANC-1 cells were passaged every 3 days (at approximately 75% confluence) by enzymatic dissociation (0.5% Trypsin-EDTA; Life Technologies).

2.4. Generation of MSC Conditioned Media (CM)

Human BM-derived MSC, previously screened for islet regenerative capacity after transplantation *in vivo* [126] were thawed from vials cryopreserved at passage 3 and maintained in culture to passage 6 in serum-free MSC media. At each passage, conditioned media was harvested (10 mL/T75 flask) following approximately 48 h of culture, and immediately stored at -20°C. For hESC-derived cultures, equal volumes of CM from MSC at passages 3-6 were combined, and supplemented with an additional 30% fresh serum free MSC media (STEMPRO® MSC SFM; Life Technologies).

2.5. Quantification of PANC-1 cell proliferation

Human ductal epithelial carcinoma cells (PANC-1) were plated at a density of 8,820 cells/cm² (in 96 well plates; 3,000 cells/well) and cultured in DMEM + 10% FBS for 4 h to permit cell adhesion. Media was then replaced with MSC-CM, DMEM + 10% FBS (positive control), unconditioned serum free MSC media + supplement, or unconditioned serum free MSC media without supplement (negative control). After 72 h cell proliferation was quantified using the CyQUANT® cell proliferation assay kit (Invitrogen) according to the manufacturer's instructions.

2.6. Gene expression analysis

Total RNA was isolated from cell cultures at specified time points using TRIzol® Reagent (Life Technologies) according to the manufacturer's instructions and quantified by spectrophotometry on a Nanodrop 2000 (Thermo Scientific). cDNA was reverse transcribed from 0.5 µg of input RNA with the iScript cDNA synthesis kit (Bio-Rad Laboratories). mRNA expression was assessed by quantitative real-time PCR using the BioRad CFX384. PCR reactions were performed in triplicate using 2.5 ng of cDNA per reaction and 312 nM forward and reverse primers (sequences provided in **Table 2.1**) with 2X SsoFast EvaGreen Supermix (Bio-Rad Laboratories) for 40 cycles of amplification (95°C 10 sec melt; 60°C 30 sec annealing/elongation). For phenotypic analysis, gene specific primers were designed to attain efficiency values between 90-110% and melt curve analysis was used to confirm primer specificity. mRNA expression was determined by relative quantification ($\Delta\Delta C_t$) corrected for input using the housekeeper gene Beta-glucuronidase (GUSB) and normalized relative to undifferentiated hESC.

Table 2.1. Primer sequences.

Gene		Sequence (5' to 3')
AMY	Forward	AATACACAACAACAAGGACGGACATC
	Reverse	TCCAAATCCCTTCGGAGCTAAA
βGEO	Forward	CCTGCTGATGAAGCAGAACA
	Reverse	TTGGCTTCATCCACCACATA
CCK_BR	Forward	TTCACCAGAACGGGCGTTG
	Reverse	TGGAAGTTGCACGTAGCAGC
CER1	Forward	ACAGTGCCCTTCAGCCAGACT
	Reverse	ACAATACTTTTTCACAGCCTTCGT
CK19	Forward	TGAGTGACATGCGAAGCCAA
	Reverse	TCAATTCTTCAGTCCGGCTGGT
CXCR4	Forward	CACCGCATCTGGAGAACCA
	Reverse	GCCCATTTCCTCGGTGTAGTT
DLX5	Forward	CGCCTCGCTGGGATTG
	Reverse	CTTGATCTTGGATCTTTTGTCTGAA
GCG	Forward	AAGCATTTACTTTGTGGCTGGATT
	Reverse	TGATCTGGATTTCTCCTCTGTGTCT
GUSB	Forward	ACGCAGAAAATATGTGGTTGGA
	Reverse	GCACTCTCGTCGGTGACTGTT
INS	Forward	AAGAGGCCATCAAGCAGATCA
	Reverse	CAGGAGGCGCATCCACA
NANOG	Forward	TGATTTGTGGGCCTGAAGAAA
	Reverse	GAGGCATCTCAGCAGAAGACA
NGN3- att (cloning)	Forward	GGGGACAAGTTTGTACAAAAAAGCAGGCTGCATGACGC CTCAACCCTCGGG
	Reverse	GGGGACCACTTTGTACAAGAAAGCTGGGTTTCACAGAA AATCTGAGAAAG
NKX2.2	Forward	CGGGCCGAGAAAGGTATGG
	Reverse	CTTTGAGCGCGTGACATGG
NKX2.5	Forward	CCCAGCCAAGGACCCTAGA
	Reverse	GCGTTGTCCGCCTCTGTCT
NKX6.1	Forward	CACACGAGACCCACTTTTTCC
	Reverse	CCCAACGAATAGGCCAAACG
OCT4	Forward	TGGGCTCGAGAAGGATGTG
	Reverse	GCATAGTCGCTGCTTGATCG
PAX4	Forward	GGGTCTGGTTTTCCAACAGAAG
	Reverse	TCAGCCCCTGGGAAGCA
PAX6	Forward	CCAGAAAGGATGCCTCATAAAGG
	Reverse	TCTGCGCGCCCCTAGTTA
PDX1	Forward	AAGTCTACCAAAGCTCACGCG
	Reverse	GTAGGCGCCGCCTGC

PECAM	Forward	GGAAAAGGCCCAATACACTT
	Reverse	TAAAACGCGGTCCTGTTCTT
SOX3	Forward	CCAGTCGTGTCGCGTCTGT
	Reverse	GCACACCTGGCTATAAATTAACATTG
SST	Forward	CCCCAGACTCCGTCAGTTTC
	Reverse	TCCGTCTGGTTGGGTTTCAG

PB-NGN3 transgene expression was quantified in clonal hESC populations (SOX17ER^{T2}; PB-NGN3) by absolute quantifications. Primers were designed to amplify a 112 bp sequence specific to the β GEO transgene (**Table 2.1**). mRNA concentrations values of SOX17-ER^{T2}; PB-NGN3 clonal cell lines were quantified using a 6 point standard curve generated from the PB-NGN3 construct (start = 33 ng, 1:10 serial dilutions).

2.7. Immunostaining

Cells were fixed in 4% (w/vol) paraformaldehyde (PFA) for 10 min at room temperature, rinsed twice in phosphate buffered saline (PBS), and blocked for 1 h in PBST (PBS + 0.1% Triton X-100 (Sigma)) + 5% species specific serum (blocking buffer). Primary antibodies diluted in blocking buffer were incubated overnight at 4°C followed by secondary antibody incubation for 1 h at room temperature. For nuclear staining, 1:1000 Hoechst 33258 (Sigma Aldrich) was added in the last 10 min of the secondary antibody incubation period. The following antibodies and dilutions were used: anti-NGN3 1:100 (Santa Cruz, SC-13793), anti-ISL1 1:50 (University of Iowa Hybridoma Bank, clone 39.4D5), anti-PAX6 1:100 (University of Iowa Hybridoma Bank, clone PAX6), anti-INS 1:350 (Sigma-Aldrich, Inc., I2018), anti-SST 1:200 (Beta Cell Biology Consortium, AB1985), Alexa Fluor 488 donkey anti-goat IgG 1:400 (Molecular Probes), Alexa Fluor 488 goat anti-mouse IgG 1:400 (Molecular Probes) and Texas Red® horse anti-mouse (1:200).

Imaging was performed using a Leica DMI6000B Inverted Microscope equipped with a Leica DFC360 FX High Resolution Camera. Images were analyzed in Leica

Application Suite Advanced Fluorescence (LAS AF) software. The mouse β TC6 cell line (β cells expressing T-antigen of simian virus 40 (SV40) [153]) was used as a positive control (Appendix 1), and cells incubated without primary antibody were used as negative control (Appendix 2) to assess the efficiency of staining. Although β TC6 cells are primarily a β insulinoma cell line, they are also known to contain cells that express small amounts of somatostatin protein [153], and therefore served as an appropriate positive control for all antibodies used in this study. For all immunostaining, the exposure time for each fluorescence channel was optimized using β TC6 cells, and the exposure time was standardized accordingly. Image acquisition parameters for each antibody including exposure and gain settings were kept constant to facilitate direct comparison between images.

2.8. β -galactosidase staining

Cells were fixed in 0.25% (vol/vol) glutaraldehyde for 10 min at room temperature and rinsed twice in PBS. Cells were incubated with X-gal solution (0.2% (w/vol) X-gal + 2mM MgCl_2 + 5mM $\text{K}_4\text{Fe}(\text{CN})_6$ + 5mM $\text{K}_3\text{Fe}(\text{CN})_6$) overnight at room temperature. Imaging was performed using Leica DMI6000B Inverted Microscope equipped with a Leica DFC295 Colour Camera. Images were analyzed in Leica Application Suite Advanced Fluorescence (LAS AF) software.

2.9. Statistical analysis

Statistical analyses were performed using GraphPad Prism version 6. All data were expressed as mean \pm SEM, and appropriate statistical tests were chosen based on

the number of groups and the number of independent variables (see figure legends for detail). $p < 0.05$ was considered significant.

CHAPTER 3:

Results

3.1. Characterization of CA2 SOX17^{ERT2}; PB-NGN3 clonal cell lines

To generate hESC lines with inducible expression of both SOX17 and NGN3, the piggyBac transposon system was used to introduce doxycycline-inducible NGN3 expression to a cell line carrying the SOX17-ER^{T2} transgene (**Figure 3.1A**). G418-resistant clonal cell populations were isolated and expanded (12 clones total). Expression of the PB-NGN3 construct was confirmed in each clonal cell line by quantifying mRNA expression of the β GEO element (transcribed along with NGN3 from the tetO promoter) following 48 h treatment of cells with DOX (0.5 μ g/ml) (**Figure 3.1B**). In all SOX17-ER^{T2}; PB-NGN3 clonal cell lines, transgene expression was exclusively detected following DOX treatment, confirming the functionality of the piggyBac system. Based on absolute qRT-PCR quantification of the PB-NGN3- β GEO transcript, clones were designated as either high or low PB-NGN3 (**Figure 3.1B**). All subsequent experiments were conducted using one representative SOX17-ER^{T2}/PB-NGN3 low cell line (clone F) and one representative SOX17-ER^{T2}; PB-NGN3 high cell line (clone G).

In order to verify protein induction, X-gal staining was performed to detect β -galactosidase activity (**Figure 3.1C**). β -galactosidase activity was detected in both SOX17-ER^{T2}; PB-NGN3^{LOW} and SOX17-ER^{T2}/PB-NGN3^{HIGH} cells following DOX treatment. Furthermore, NGN3 protein induction in representative low and high clonal cell lines was verified by immunocytochemistry (**Figure 3.2A and B**). Like β -galactosidase activity, NGN3 protein induction was only detected in both clonal cell lines following DOX treatment.

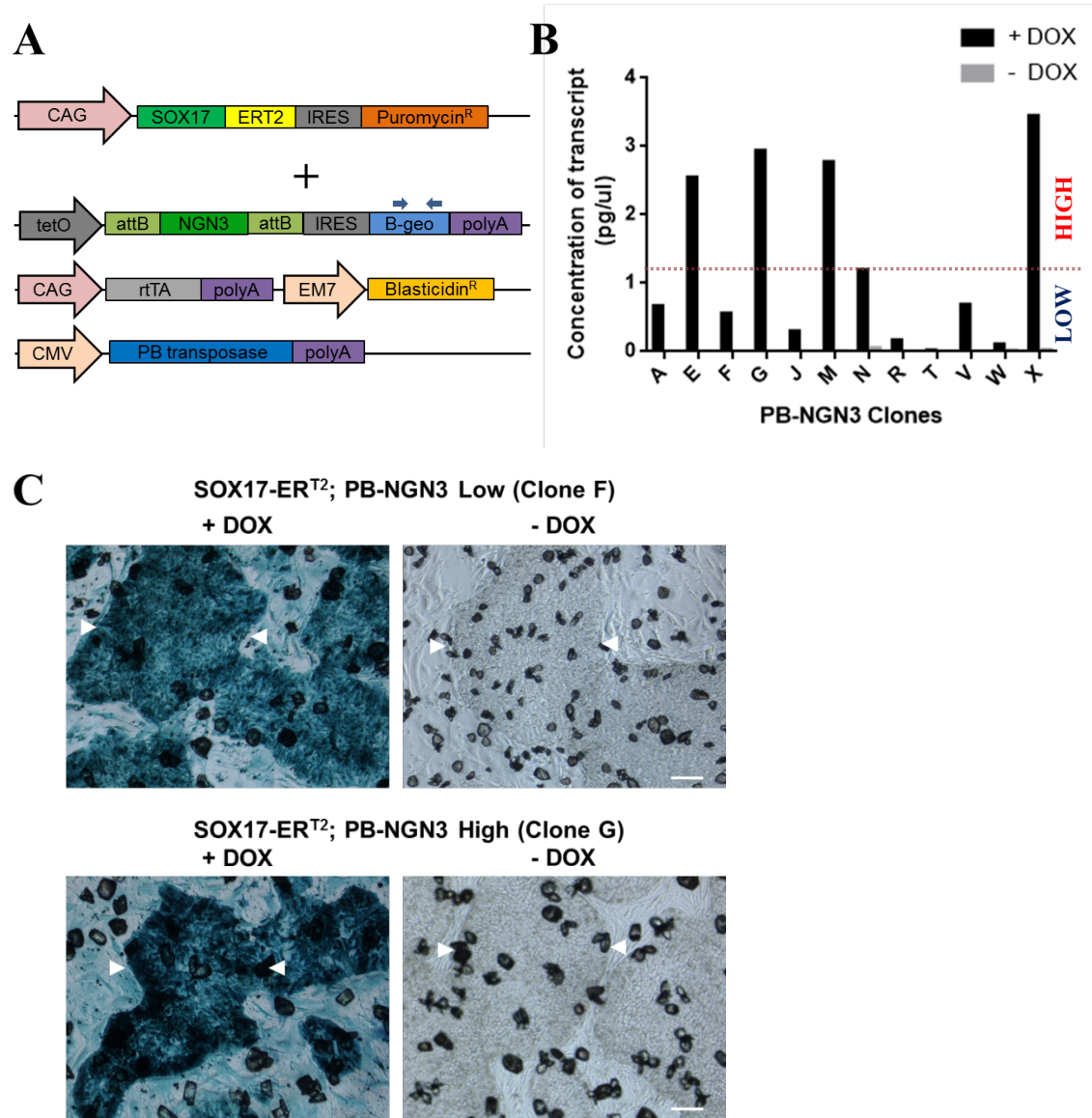


Figure 3.1. Characterization of clonal hESC lines with inducible NGN3 expression. A) Schematic illustration of the SOX17-ER^{T2} and piggyBac (PB)-NGN3-βGEO transgene expression systems. βGEO-specific primer annealing sites used to quantify PB transgene expression are indicated by blue arrows. B) Quantification of PB transgene expression by qRT-PCR in SOX17-ER^{T2}; PB-NGN3 clonal cell lines with or without DOX treatment (48 h). C) X-gal staining to detect β-galactosidase activity in SOX17-ER^{T2}; PB-NGN3 low and high cell lines with or without DOX treatment (72 h). Scale bar equals 100μm. White arrowheads indicate cell colonies.

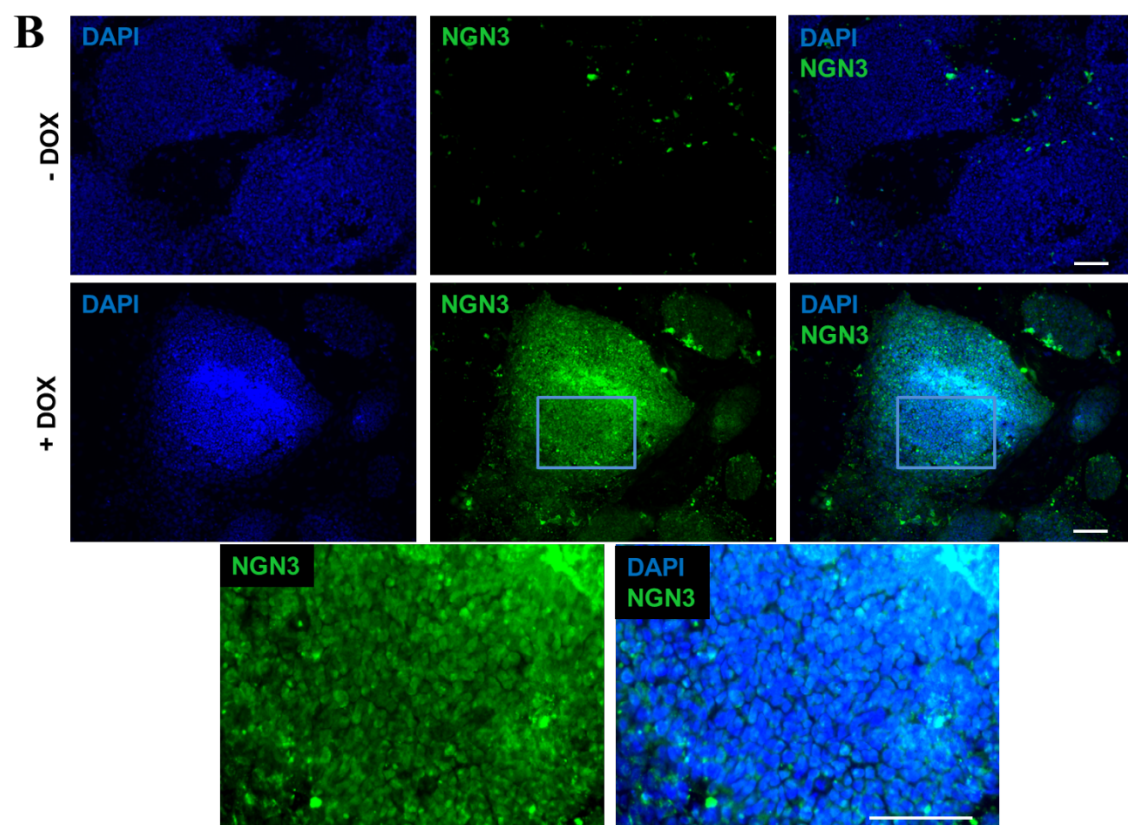
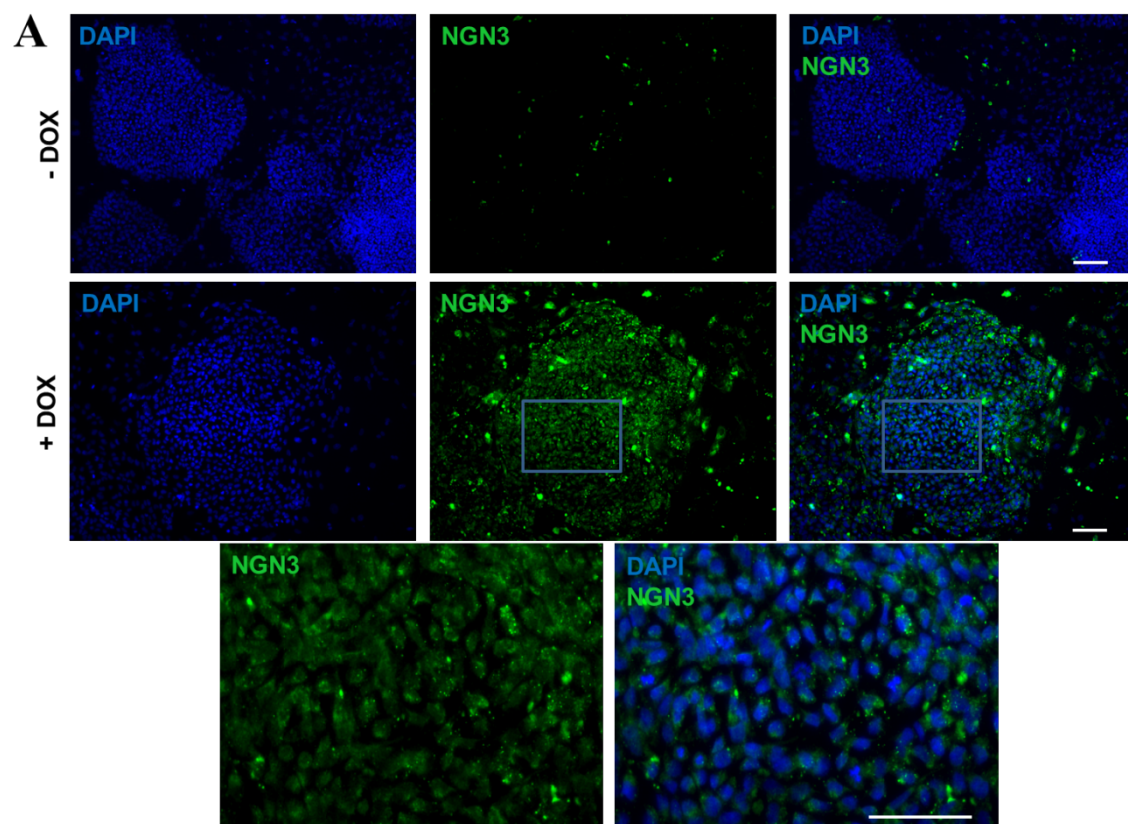


Figure 3.2. Immunolocalization of NGN3. Immunocytochemistry of NGN3 in A) SOX17-ER^{T2}; PB-NGN3 low and B) SOX17-ER^{T2}; PB-NGN3 high clonal cell lines with or without DOX treatment (72 h). Scale bar equals 100µm.

3.2. Differentiation of pluripotent hESCs to definitive endoderm progenitors (DEP)

Based on our preliminary data and previous description of stepwise pancreatic differentiation [136], SOX17-ER^{T2}; PB-NGN3^{LOW/HIGH} cells were subjected to an initial 5 days of SOX17 activation to initiate DE differentiation. To confirm that this period was sufficient for DEP specification, SOX17-ER^{T2}; PB-NGN3^{LOW} and SOX17-ER^{T2}; PB-NGN3^{HIGH} cells were harvested following 5 days SOX17 activation (4OHT treatment; in the absence of DOX-induction of NGN3) and the expression of DE markers was assessed. In both cell lines, 4OHT-mediated SOX17 activation significantly induced the expression of known DE markers CXCR4 and DLX5 mRNA when compared to untreated cells (**Figure 3.3**). Changes in CER1 expression in both cell lines were not significantly different from untreated cells after 5 days of 4OHT treatment (**Figure 3.3**). Collectively, these results suggest the induction of DE phenotype.

3.3. NGN3 expression directs SOX17-DEPs to a pancreatic endocrine fate

Having established our ability to drive SOX17-mediated DE differentiation, we next assessed the effect of ectopic NGN3 expression in DE progenitors. NGN3 expression was induced by DOX treatment in SOX17-ER^{T2}; PB-NGN3^{LOW} and SOX17-ER^{T2}; PB-NGN3^{HIGH} clones subsequent to 5 days of SOX17 activation, and changes in cell phenotype were assessed over 12 days. We verified that the expression of PB-NGN3 transgene was maintained during the differentiation by quantifying mRNA levels of the β GEO element after 12 days of DOX treatment (0.5 μ g/ml) (**Figure 3.4A**).

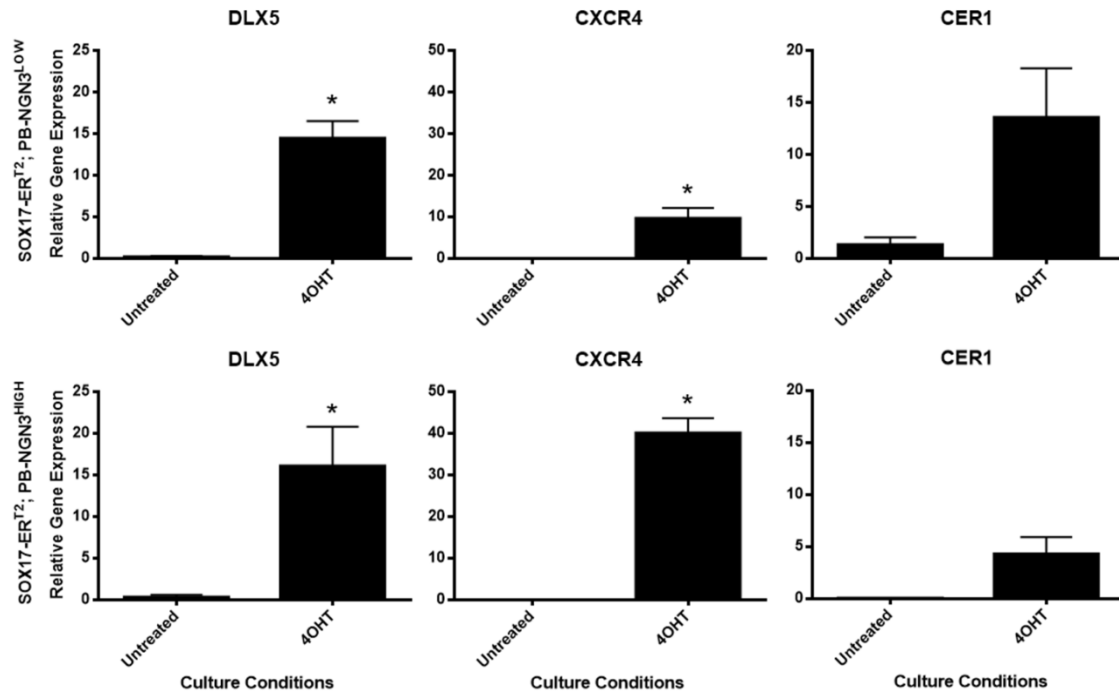


Figure 3.3. Characterization of SOX17-induced definitive endoderm differentiation in SOX17-ER^{T2};PB-NGN3 cell lines. Real-time PCR analysis of definitive endoderm markers following 5 days of SOX17 activation in SOX17-ER^{T2}; PB-NGN3^{LOW} and SOX17-ER^{T2}; PB-NGN3^{HIGH} cells. Gene expression is corrected for input based on expression of GUSB and expressed relative to undifferentiated hESC. Data are presented as the mean \pm SEM (n = 4). Statistical analysis was performed using one-tailed Student's t-test; * indicates significant difference compared to control (untreated) cells (p<0.05).

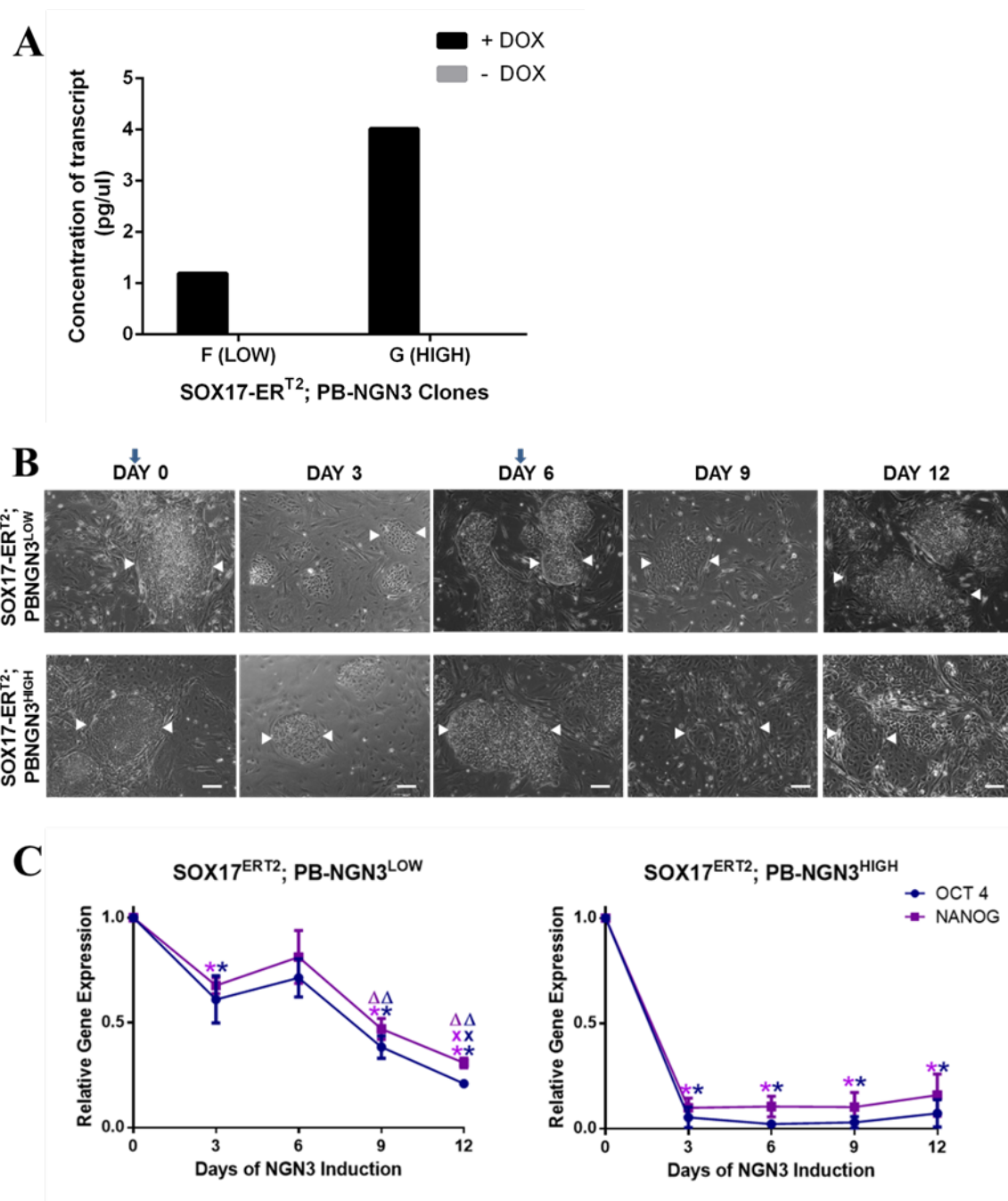


Figure 3.4. SOX17-ER^{T2}; PB-NGN3^{LOW} and SOX17-ER^{T2}; PB-NGN3^{HIGH} clones show loss of pluripotency-associated factors following NGN3 induction. A) Quantification of PB transgene expression by qRT-PCR in SOX17-ER^{T2}; PB-NGN3 clonal cell lines after 12 days of DOX treatment. B) Representative images demonstrating the morphology of SOX17-ER^{T2}; PB-NGN3^{LOW} and SOX17-ER^{T2}; PB-NGN3^{HIGH} cell lines following 3, 6, 9 or 12 days of NGN3 induction post SOX17-mediated DE induction (indicated as Day 0). As cells remained proliferative following transgene induction, cultures were passaged following 5 days of SOX17 induction (indicated as Day 0) and at day 6 (indicated by blue arrows). White arrowheads indicate individual cell colonies. Scale bar equals 100µm. C) Real-time PCR analysis of pluripotency markers OCT4 and NANOG over 12 days of NGN3 induction. Gene expression is corrected for input based on expression of GUSB and expressed relative to Day 0 of NGN3 induction (DE progenitor cells). Data are presented as the mean ± SEM (n=3). For each gene, statistical analysis was performed using One-way ANOVA with Tukey's multiple comparisons post-hoc test. * indicates significant difference (P<0.05) from expression at day 0 for each gene; X indicates significant difference from expression at day 3 for each gene; Δ indicates significant difference from expression at day 6 for each gene.

After 3 days of NGN3 induction, both SOX17-ER^{T2}; PB-NGN3^{LOW} and SOX17-ER^{T2}; PB-NGN3^{HIGH} cells retained a high nucleus to cytoplasm ratio and grew within tightly packed colonies, features characteristic of both hESC and DE progenitor cells. However, following 9 days of NGN3 induction, SOX17-ER^{T2}; PB-NGN3^{HIGH} cells appeared larger in size, and did not grow within densely packed cell colonies when compared to the starting population of DE progenitors (Day 0) (**Figure 3.4B**). In contrast, SOX17-ER^{T2}; PB-NGN3^{LOW} cells did not show overt morphological changes over the 12 days of NGN3 induction (**Figure 3.4B**).

To assess the effect of NGN3 induction on the expression of pluripotency-associated transcription factors, expression of *OCT4* and *NANOG* were assessed by qRT-PCR at multiple time points over 12 days of NGN3 induction. Both SOX17-ER^{T2}; PB-NGN3 clonal cell lines showed a significant reduction in both OCT4 and NANOG mRNA expression within 3 days of NGN3 induction. However, SOX17-ER^{T2}; PB-NGN3^{LOW} cells demonstrated a progressive reduction in pluripotency-associated gene expression over the 12 days of NGN3 induction while SOX17-ER^{T2}; PB-NGN3^{HIGH} cells showed a sharp reduction at day 3 with no subsequent change in gene expression levels from day 3 to day 12 (**Figure 3.4C**). Taken together, these data suggest that both low and high levels of NGN3 expression in SOX17-DEPs result in cell differentiation and loss of pluripotency-associated markers.

To determine whether NGN3 activation stimulated pancreatic endocrine cell differentiation, we assessed the expression of transcription factors associated with early pancreatic endocrine differentiation (PAX4, PAX6 NKX6.1 and NKX2.2), β cell phenotype (PDX1), δ cell phenotype (CCK_BR), β and δ cell phenotype (ISL1), and ductal

epithelial cell phenotype (CK19). In addition, mRNA transcript levels for pancreatic endocrine hormones insulin (INS), glucagon (GCG), somatostatin (SST) and the exocrine pancreatic enzyme amylase (AMY) were assessed using qRT-PCR. SOX17-ER^{T2}; PB-NGN3^{LOW} and SOX17-ER^{T2}; PB-NGN3^{HIGH} cells showed divergent patterns in the expression of early pancreatic endocrine markers (**Figure 3.5A**). SOX17-ER^{T2}; PB-NGN3^{HIGH} cells showed a significant induction of *PAX4* expression, a direct target of NGN3 [154] crucial for β and δ cell differentiation [91], after 3 days of DOX treatment with expression returning to baseline by day 6 (**Figure 3.5A**). SOX17-ER^{T2}; PB-NGN3^{HIGH} cells also showed early induction of *PAX6* expression, a transcriptional factor critical for the development of all pancreatic endocrine subtypes and also expressed in all mature pancreatic endocrine cells [89], after 6 days of NGN3 induction when compared to undifferentiated controls (**Figure 3.5A**). In contrast, SOX17-ER^{T2}; PB-NGN3^{LOW} cells did not show significant induction of these genes (**Figure 3.5A**). *NKX6.1* expression, a transcription factor implicated in early β and δ cell progenitor fates [96] and with mature β cell function [89,97], was significantly upregulated in both clones at day 12 of NGN3 induction (**Figure 3.5A**). Interestingly, the expression of *NKX2.2*, a TF important for terminal differentiation of β cells and suppression of the δ cell phenotype [99], was not significantly induced by ectopic NGN3 expression in either clone (**Figure 3.5A**). These data suggest that level of NGN3 expression in SOX17-DEPs is important in the induction of early pancreatic endocrine genes.

The expression of markers of mature endocrine cell types also varied between NGN3 high and low-expressing cell lines. SOX17-ER^{T2}; PB-NGN3^{HIGH} but not SOX17-ER^{T2}; PB-NGN3^{LOW} cells showed increased expression of the β cell marker PDX1 [155]

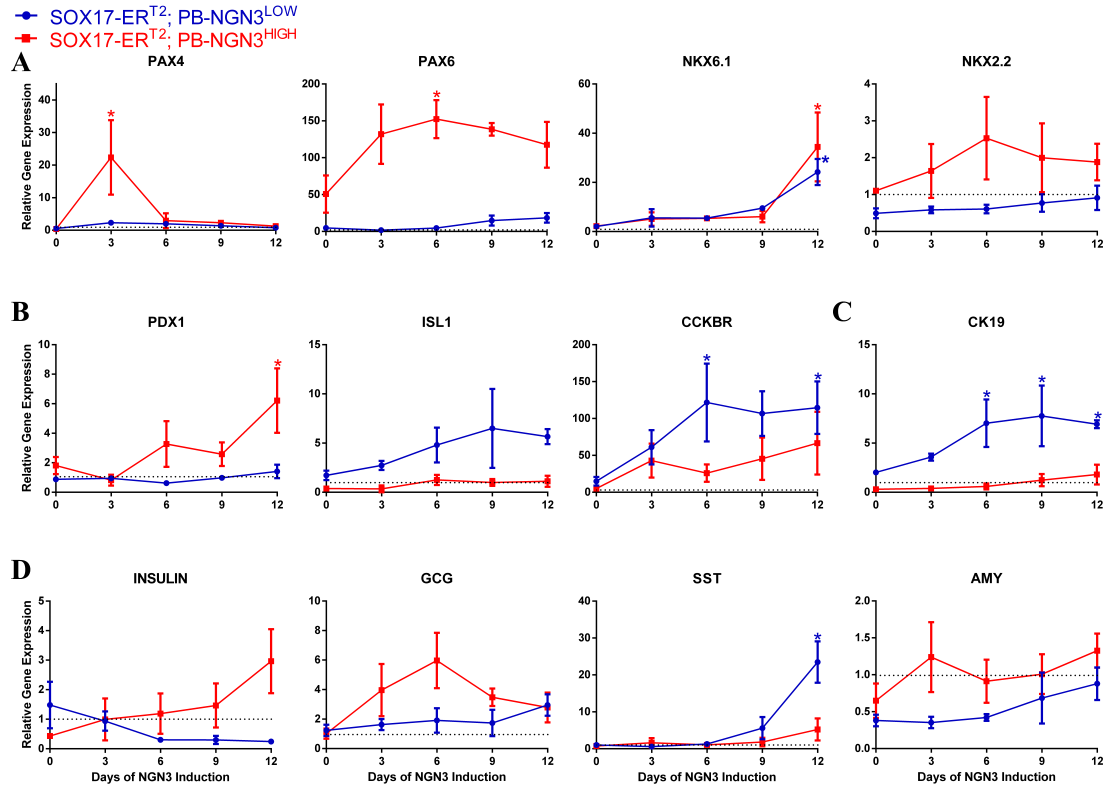


Figure 3.5. NGN3 induction in SOX17 DEPs promotes expression of pancreatic endocrine markers. Real-time PCR analysis of genes associated with pancreatic endocrine development and function over 12 days of NGN3 induction post SOX17-mediated DE differentiation (indicated as day 0). A) Markers of early pancreatic endocrine differentiation, B) markers of β cell and/or δ cell fates, C) marker of pancreatic ductal epithelia, and D) pancreatic hormones (INS, GCG, SST) and exocrine marker (AMY). Gene expression is corrected for input based on expression of GUSB and expressed relative to undifferentiated hESC. Data are presented as mean \pm SEM (n=3). Statistical analysis was performed using two-way ANOVA with Tukey's multiple comparisons post-hoc test. * indicates significant difference ($P < 0.05$) from day 0 (SOX17 definitive endoderm cells) for each cell line as indicated by colour.

after 12 days of NGN3 induction (**Figure 3.5B**). However, *INS* gene expression was not significantly upregulated in either cell population during the 12 day period of NGN3 induction (**Figure 3.5B**). In contrast, SOX17-ER^{T2}; PB-NGN3^{LOW} cells showed a significant induction of the δ cell markers *SST* and *CCK_BR* [40] (**Figure 3.5B & D**). The expression of neither the α cell marker glucagon (*GCG*) nor the exocrine pancreas marker amylase (*AMY*) [156] were induced by NGN3 induction in either cell line (**Figure 3.5D**). Interestingly, *ISL1*, a TF that co-regulates both *SST* and *INS* gene expression [102,157], was also not significantly induced in either cell line (**Figure 3.5B**). Unexpectedly, we noted the induction of *CK19* expression, a marker of pancreatic duct cells [158], following 6 days of NGN3 induction in SOX17-ER^{T2}; PB-NGN3^{LOW} cells but not in SOX17-ER^{T2}; PB-NGN3^{HIGH} cells (**Figure 3.5C**). Collectively, these data suggest that the level NGN3 expression directs endocrine pancreatic differentiation such that high NGN3 expression promoted β cell-like characteristics, while lower NGN3 expression promoted a δ cell-like expression profile with some ductal cell characteristics.

The induction of PAX6 and *ISL1* protein were subsequently assessed by immunocytochemistry in SOX17-ER^{T2}; PB-NGN3^{LOW} (**Figure 3.6 and 3.7**) and SOX17-ER^{T2}; PB-NGN3^{HIGH} (**Figure 3.8 and 3.9**) cell lines at multiple time points during 12 days of NGN3 induction following SOX17-mediated DE specification. Interestingly, in SOX17-ER^{T2}; PB-NGN3^{LOW} cells small clusters of PAX6-positive (**Figure 3.6**) and *ISL1*-positive (**Figure 3.7**) cells were detected after 9 and 12 days of NGN3 induction even though changes in gene expression were not significant (**Figure 3.5**). We likewise observed clusters of SOX17-ER^{T2}; PB-NGN3^{HIGH} cells showing convincing nuclear localization of PAX6 (**Figure 3.8**) and *ISL1* (**Figure 3.9**) after 9 and 12 of NGN3

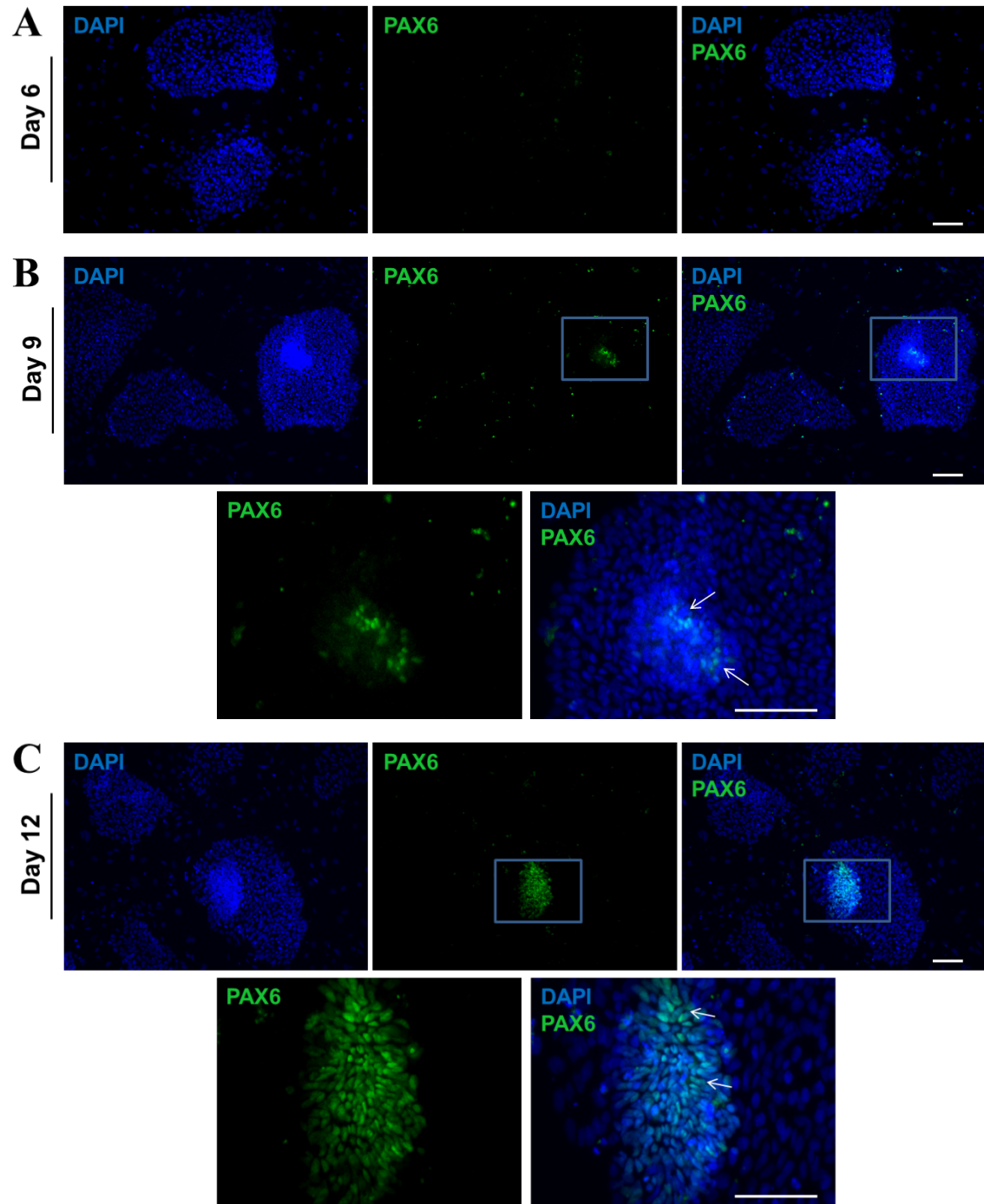


Figure 3.6. NGN3 induction in SOX17-ER^{T2}; PB-NGN3^{LOW} cells induces areas of PAX6 positive cells. Representative images demonstrating immunocytochemistry of the pancreatic endocrine development transcription factor PAX6 after A) 6, B) 9 and C) 12 days of NGN3 induction post SOX17-mediated DE differentiation. Areas with positive immunoreactivity were magnified (indicated by boxes). Arrows indicate areas positive for nuclear localization of PAX6. Scale bar equals 100 μ m. n = 3.

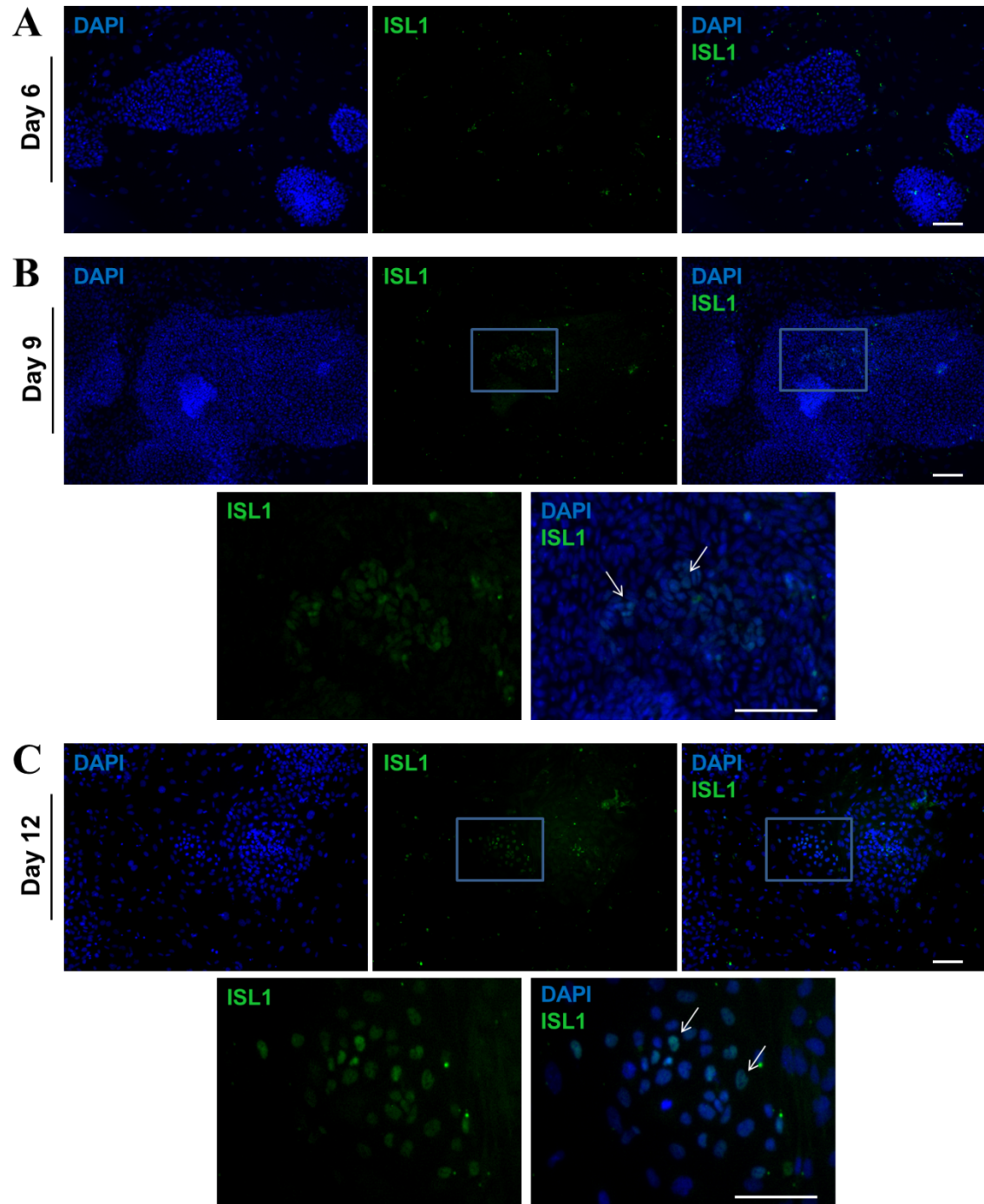


Figure 3.7. NGN3 induction in SOX17-ER^{T2}; PB-NGN3^{LOW} cells induces areas of ISL1 positive cells. Representative images demonstrating immunocytochemistry of the pancreatic endocrine development transcription factor ISL1 after A) 6, B) 9 and C) 12 days of NGN3 induction post SOX17-mediated DE differentiation. Areas with positive immunoreactivity were magnified (indicated by boxes). Arrows indicate representative areas positive for nuclear localization of ISL1. Scale bar equals 100 μ m. n = 3.

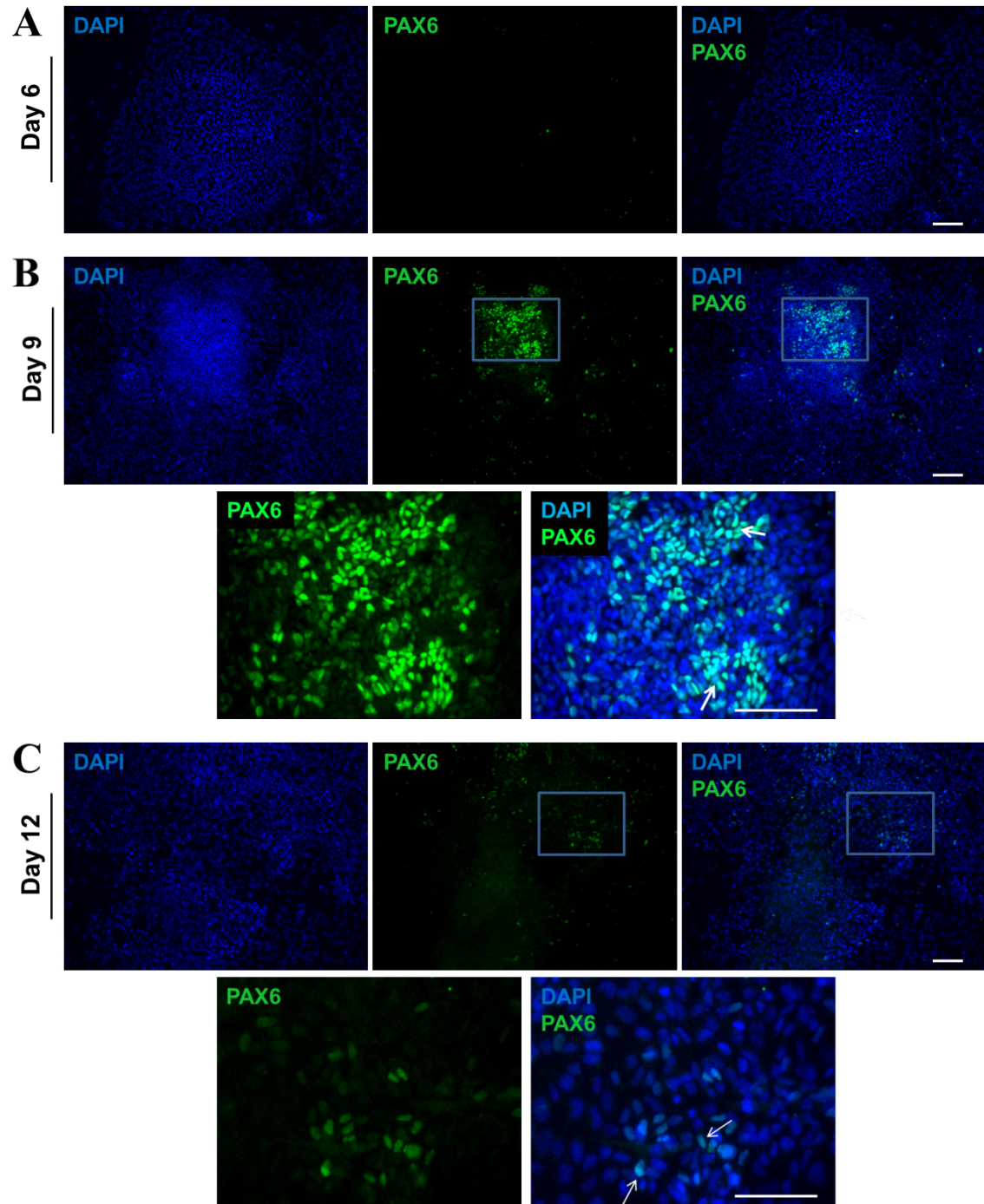


Figure 3.8. NGN3 induction in SOX17-ER^{T2}; PB-NGN3^{HIGH} induces areas of PAX6 positive cells. Representative images demonstrating immunocytochemistry of the pancreatic endocrine development transcription factor PAX6 after A) 6, B) 9 and C) 12 days of NGN3 induction post SOX17-mediated DE differentiation. Areas with positive immunoreactivity were magnified (indicated by boxes). Arrows indicate representative areas positive for nuclear localization of PAX6. Scale bar equals 100µm. n = 3.

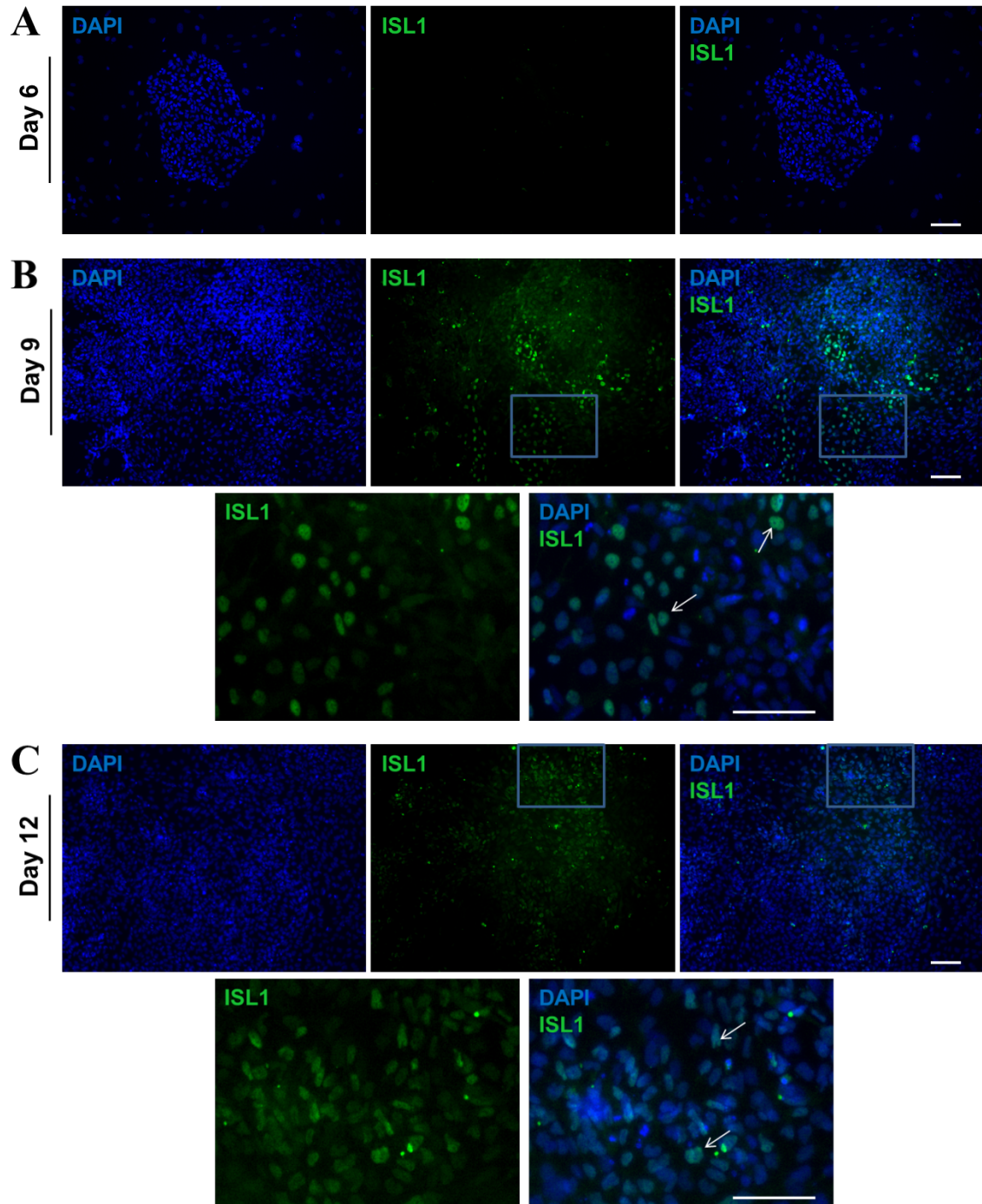


Figure 3.9. NGN3 induction in SOX17-ER^{T2}; PB-NGN3^{HIGH} cells induces areas of ISL1 positive cells. Representative images demonstrating immunocytochemistry of the pancreatic endocrine development transcription factor ISL1 after A) 6, B) 9 and C) 12 days of NGN3 induction post SOX17-mediated DE differentiation. Areas with positive immunoreactivity were magnified (indicated by boxes). Arrows indicate representative areas positive for nuclear localization of ISL1. Scale bar equals 100 μm. n = 3.

induction. However, we observed heterogeneity in both clonal cell lines, with a significant number ISL1 and PAX6-negative cells observed at both day 9 and 12 of NGN3 induction. Although we did not observe a homogeneous population of differentiated cells, we noted that the proportion of positive cells was greater in later stages of NGN3 induction (days 9 and 12) than at earlier stages (day 6). Considering that PAX6 and ISL1 are crucial for the development and specification of all islet cells [95,159], these data collectively suggest that expression of NGN3 in SOX17-DEPs directs a pancreatic endocrine phenotype.

We also performed immunocytochemistry for INS (**Figure 3.10**) and SST (**Figure 3.11 and 3.12**) in both SOX17-ER^{T2}; PB-NGN3 cell lines after 12 days of NGN3 expression. We did not detect INS protein in either clone during the course of NGN3 induction. These data support our findings showing INS mRNA expression was not significantly induced in either SOX17-ER^{T2}; PB-NGN3^{LOW} or SOX17-ER^{T2}; PB-NGN3^{HIGH} cells during the course of NGN3 induction (**Figure 3.5**). Also, we did not detect convincing cytoplasmic immunofluorescence for SST in SOX17-ER^{T2}; PB-NGN3^{LOW} cells over 12 days of NGN3 induction (**Figure 3.11**), even though we detected a significant induction of SST mRNA by day 12 of NGN3 expression (**Figure 3.5**). Similarly, we did not detect convincing cytoplasmic staining for SST in SOX17-ER^{T2}; PB-NGN3^{HIGH} cells over 12 days of NGN3 induction (**Figure 3.12**). The fluorescence detected in both SOX17-ER^{T2}; PB-NGN3 clones is not characteristic of positive cytoplasmic hormone staining as detected by SST immunocytochemistry in β TC6 cells (**Figure 3.11A**).

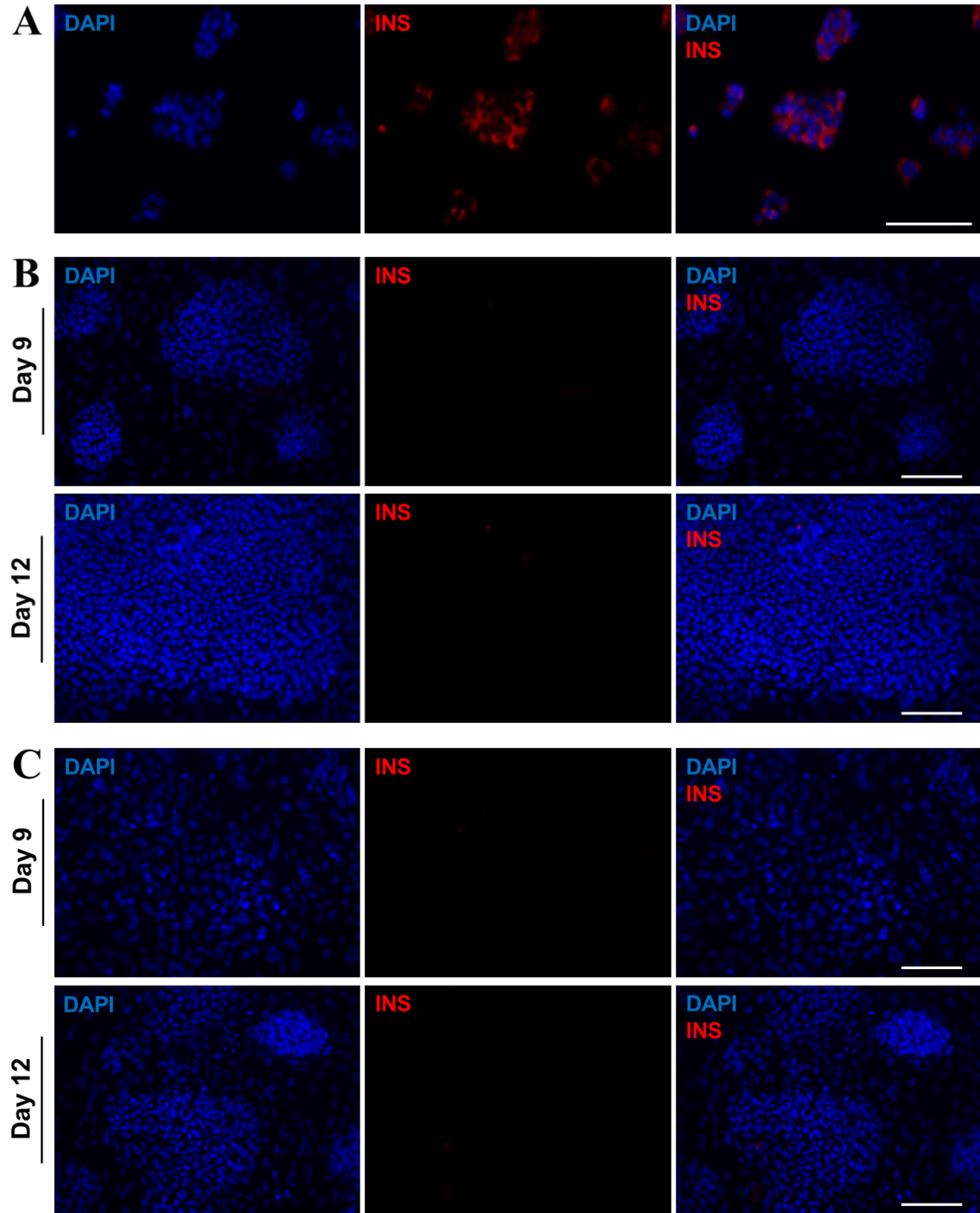


Figure 3.10. SOX17-ER^{T2}; PB-NGN3 cells do not express insulin protein after 12 days of NGN3 induction. Immunocytochemistry of insulin in: A) β TC6 cells as positive control, and B) SOX17-ER^{T2}; PB-NGN3^{LOW} and C) SOX17-ER^{T2}; PB-NGN3^{HIGH} cells after 9 and 12 days of NGN3 induction. Scale bar equals 100 μ m. n = 4.

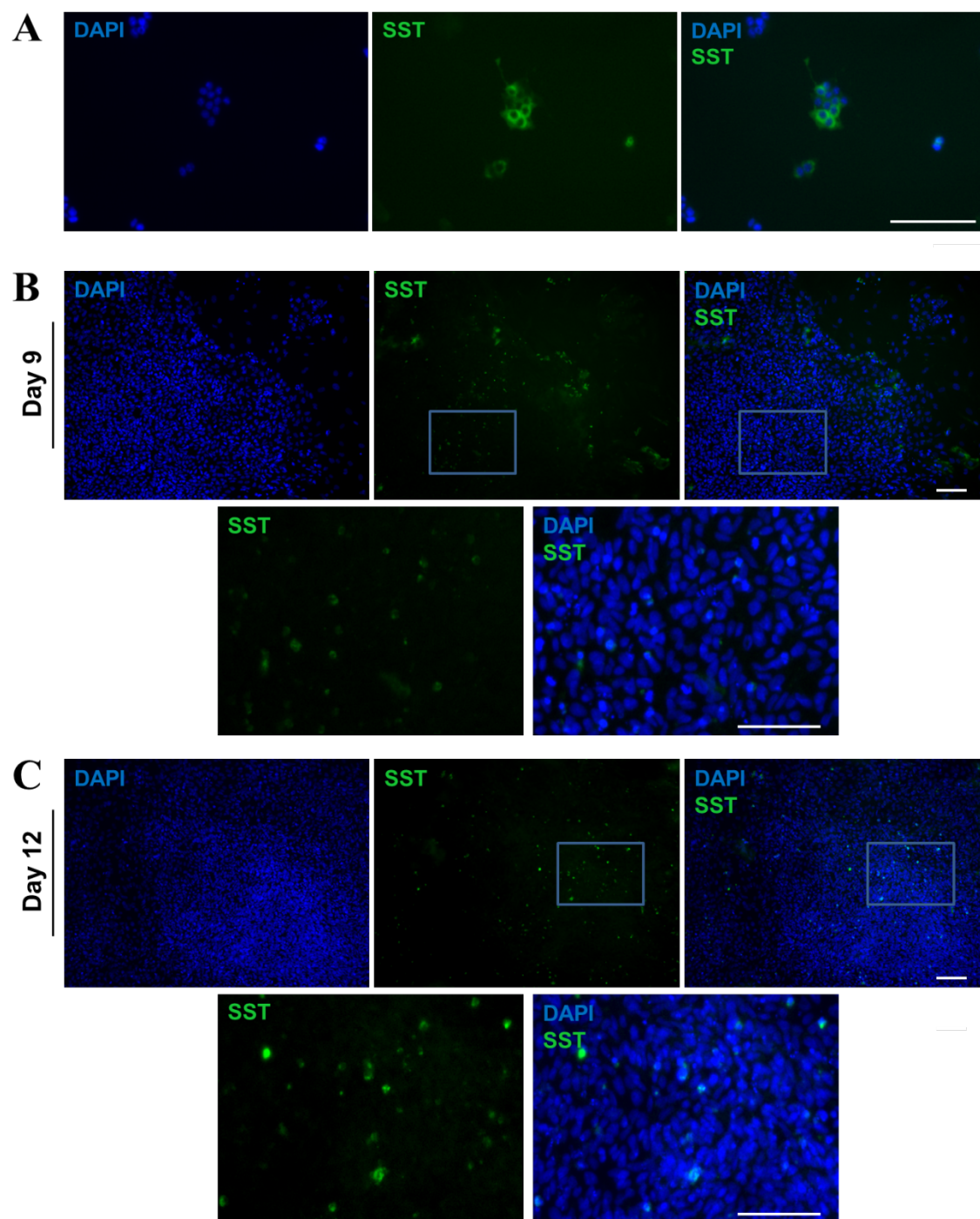


Figure 3.11. SOX17-ER^{T2}; PB-NGN3^{LOW} cells do not express SST protein after 12 days of NGN3 induction. Representative images demonstrating immunocytochemical detection of SST in: A) β TC6 cells as positive control, and SOX17-ER^{T2}; PB-NGN3^{LOW} cells after B) 9 and C) 12 days of NGN3 induction. Areas with positive immunoreactivity were magnified (indicated by boxes). Scale bar equals 100 μ m. n = 3.

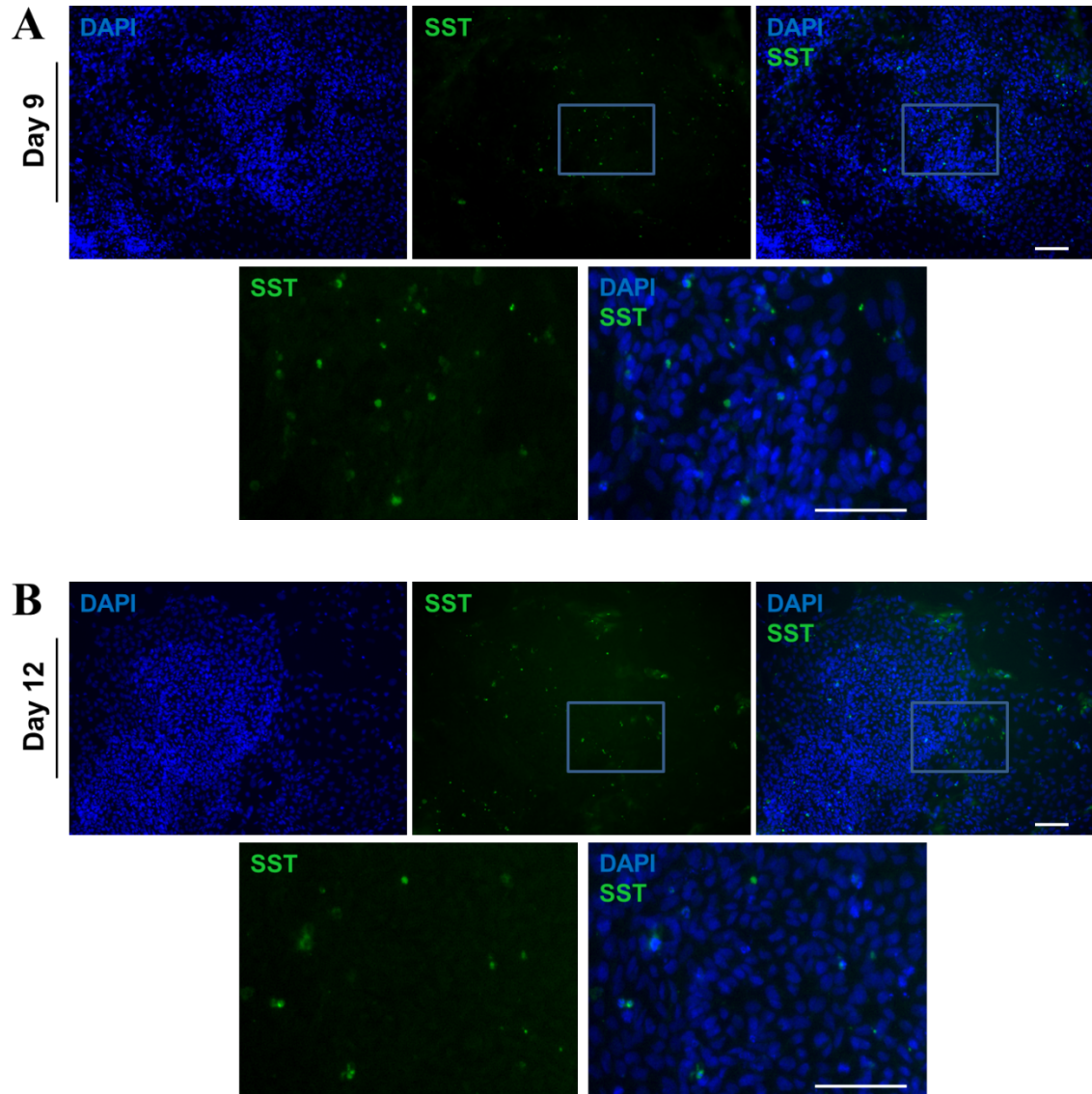


Figure 3.12. SOX17-ER^{T2}; PB-NGN3^{HIGH} cells express do not express SST protein after 12 days of NGN3 induction. Representative images demonstrating immunocytochemical detection of SST in: SOX17-ER^{T2}; PB-NGN3^{HIGH} cells after A) 9 and B) 12 days of NGN3 induction. Areas with apparent immunoreactivity were magnified (indicated by boxes). Scale bar equals 100 μ m. n = 3.

We also attempted immunocytochemistry for NKX2.2 and NKX6.1 but were unable to obtain convincing images due to high levels of non-specific background staining.

3.4. Development of *in vitro* strategy to assess the effects of MSC-secreted factors on the differentiation of SOX17-ER^{T2}; PB-NGN3 cells

In order to determine the effects of MSC-conditioned media on our SOX17-ER^{T2}; PB-NGN3 endocrine precursor populations, it was necessary to first assess whether the serum-free MSC media would support hESC growth without inducing spontaneous differentiation. Therefore, we cultured undifferentiated SOX17-ER^{T2} hESCs (no 4OHT treatment) in serum-free MSC media for 5 days and compared them to cells maintained for the same period in standard culture conditions (hESC media). Cells maintained under either culture condition demonstrated morphological characteristics associated with pluripotent hESC, such as high nuclear to cytoplasmic ratio and dense colonies with sharp contrasting borders (**Figure 3.13A**). No morphological signs of spontaneous differentiation were observed for either condition. We next assessed the expression of pluripotency markers (OCT4, NANOG), a neuroectodermal marker (SOX3), early mesoderm marker (NKX2.5), and endothelial marker (PECAM) in undifferentiated SOX17-ER^{T2} cells and SOX17-ER^{T2} cells treated with 4OHT over 5 days culture in hESC media or MSC media (**Figure 3.13B**). Under both conditions, cells maintained expression of OCT4 and NANOG mRNA, as expected for both undifferentiated hESC and DEPs. Furthermore, no induction of PECAM or NKX2.5 was detected under either condition, suggesting the absence of mesodermal differentiation (**Figure 3.13B**). Culture of undifferentiated SOX17-ER^{T2} cells or SOX17-DEPs in MSC

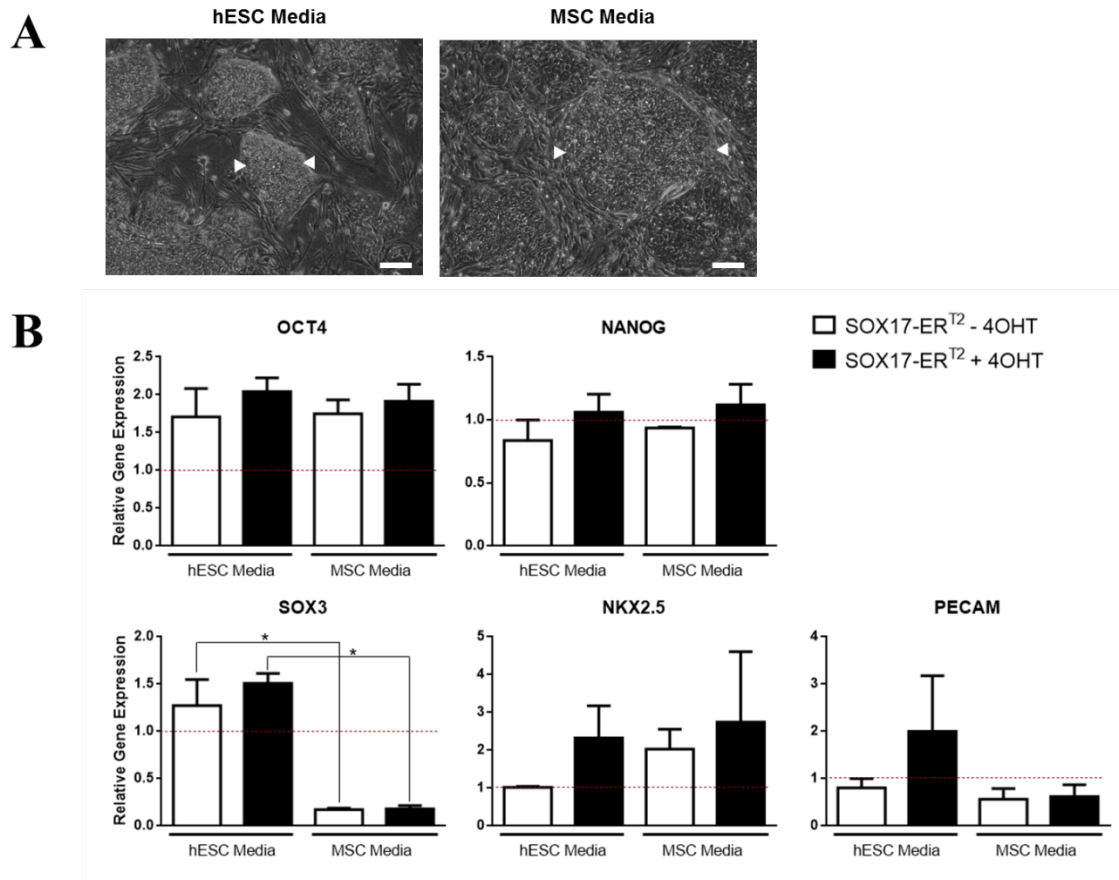


Figure 3.13. Culture of hESC and SOX17-DEPs in serum-free MSC media does not promote spontaneous differentiation. A) Representative images of undifferentiated SOX17-ER^{T2} cells cultured in either hESC media or MSC serum-free media. Scale bar equals 100µm; arrowheads indicate individual cell colonies. B) Real time-PCR analysis of pluripotent markers (OCT4, NANOG), ectodermal (SOX3) and mesodermal markers (NKX2.5, PECAM) expression in SOX17-ER^{T2} cells with or without 4OHT treatment (0.2 µM) grown in either hESC media or MSC serum-free media for 5 days. Gene expression is corrected for input based on expression of GUSB and expressed relative to undifferentiated hESC. Data are presented as the mean ± SEM (n = 3). Statistical analysis was performed using one-tailed Student's t-test; * indicates significant difference between cells cultured in hESC media and MSC media (p<0.05).

media showed a significant decrease in the expression of SOX3 compared to cells maintained in hESC media (**Figure 3.13B**), suggesting the possible suppression of neuroectodermal lineage potential. Nonetheless, SOX17-ER^{T2} cells cultured in MSC media showed no sign of spontaneous differentiation based on the analyses performed.

3.5. Islet-regenerative MSC-conditioned media promoted PANC-1 cell proliferation *in vitro*

Previous studies characterizing the islet-regenerative potential of human bone-marrow derived MSCs were performed using MSCs expanded in culture conditions containing bovine serum [121,126]. Since bovine serum is known to induce the spontaneous differentiation of hESCs, MSCs were propagated in serum-free media to generate conditioned media (CM) for hESC experiments. Consequently, we assessed whether MSC retained their proliferation-inducing characteristics when cultured under serum-free conditions based on their previously established ability to promote the proliferation of PANC-1 human pancreatic carcinoma cells *in vitro* [121]. As previously observed under serum containing conditions [121], PANC-1 cells showed a significant induction in cell proliferation when cultured in CM generated by regenerative MSC compared to non-regenerative MSC, serum-free MSC media controls, or PANC-1 cell basal media (DMEM + 10% bovine serum) (**Figure 3.14B**). Thus, conditioned media generated from regenerative MSCs generated under serum-free conditions retained proliferative-inducing capacity on PANC-1 cells *in vitro*.

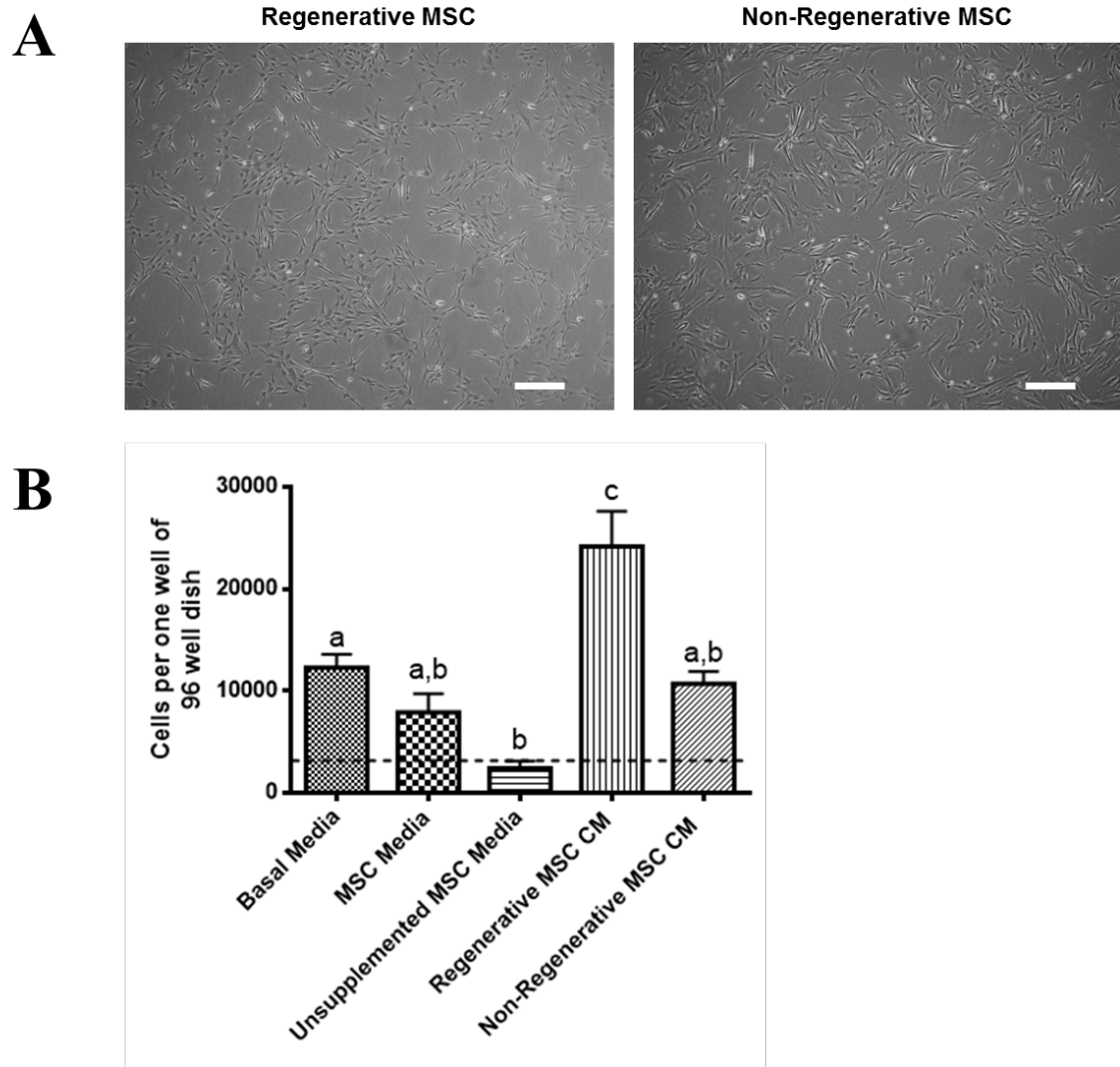


Figure 3.14. Islet-regenerative MSC-conditioned media promotes PANC-1 cell proliferation. A) Representative images of regenerative and non-regenerative MSC morphology 24h after passaging (passage 4). Scale bar equals 100 μ m. B) PANC1 cell proliferation as quantified using the Cyquant cell proliferation assay. PANC-1 cells were cultured in either basal media (DMEM + 10% FBS; positive control), unsupplemented MSC media (negative control), MSC serum-free media or MSC-conditioned media from regenerative or non-regenerative MSCs. Data are presented as the mean \pm SEM (n = 4). Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons post-hoc test. Statistical significance ($p < 0.05$) is indicated by the use of different letters between groups; same letters indicate that groups are not significantly different from each other.

3.6. Culture of SOX17-ER^{T2}; PB-NGN3 cells in regenerative MSC-CM does not promote pancreatic endocrine maturation

In order to assess the effects of islet regenerative MSC-CM on endocrine cell differentiation *in vitro*, SOX17-ER^{T2}; PB-NGN3^{LOW} and SOX17-ER^{T2}; PB-NGN3^{HIGH} cells were cultured in regenerative MSC-CM, non-regenerative MSC-CM or basal MSC media alone during NGN3-mediated specification of SOX17-DE progenitors. The expression of genes associated with pancreatic endocrine development and function were subsequently quantified by qRT-PCR. We first assessed differentiation when SOX17-ER^{T2}; PB-NGN3 cells were cultured in MSC-CM (or control conditions) from days 3-12 of NGN3 induction, following 5 days of SOX17-mediated DE differentiation. SOX17-ER^{T2}; PB-NGN3^{LOW} cells cultured in islet-regenerative MSC-CM from days 3-12 of NGN3 induction did not show induction of markers of pancreatic endocrine cell maturation such as PDX1, ISL1, INS or SST compared to cells maintained in non-regenerative MSC-CM or MSC media alone (**Figure 3.15**). In fact, we observed a significant reduction of *NKX6.1* and *SST* gene expression in regenerative MSC-CM culture when compared to NGN3-induction alone, suggesting suppression of endocrine differentiation (**Figure 3.15**). Similarly, SOX17-ER^{T2}; PB-NGN3^{HIGH} cells cultured in islet-regenerative MSC-CM from day 3 to day 12 of culture did not significantly alter the expression of pancreatic endocrine markers compared to cells maintained in non-regenerative MSC-CM or MSC media alone (**Figure 3.16**).

We next assessed the effects of islet regenerative MSC-CM on SOX17-ER^{T2}; PB-NGN3 cell differentiation during later stages of NGN3 induction (conditioned media added at days 6-12 or at days 9-12). Interestingly, SOX17-ER^{T2}; PB-NGN3^{LOW} cells

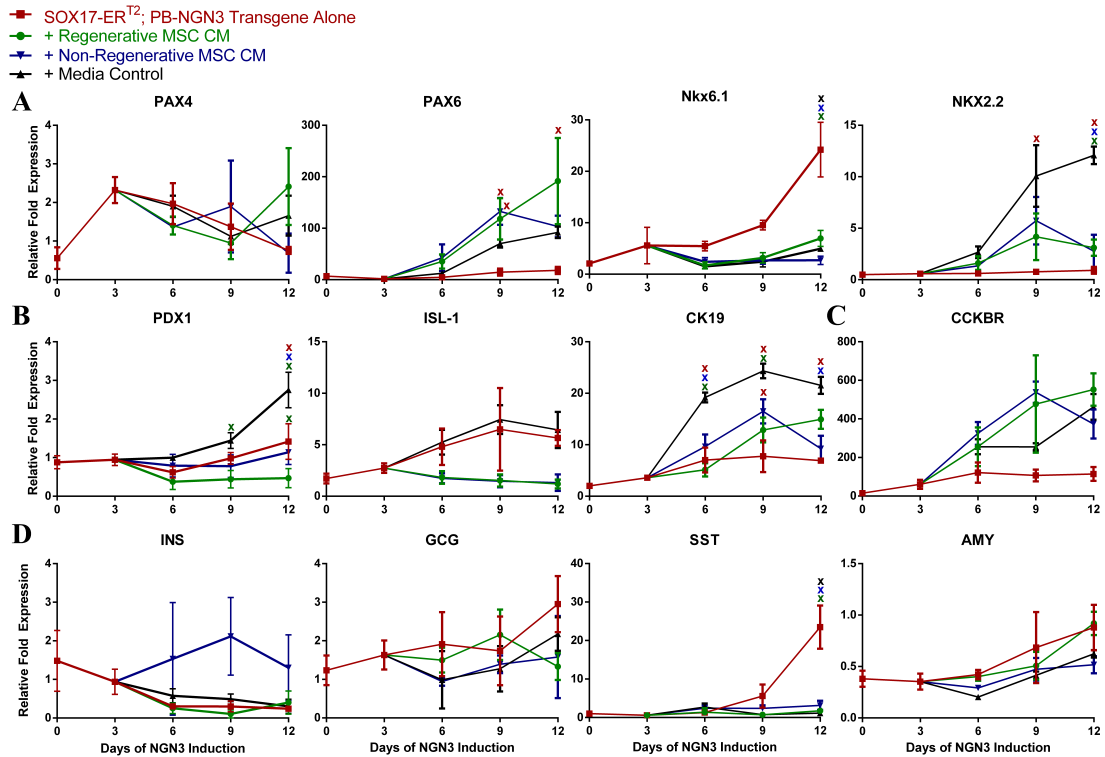


Figure 3.15. Culture of SOX17-ER^{T2}; PBNGN3^{Low} cells in regenerative MSC conditioned media (CM) does not promote pancreatic endocrine maturation. Real time-PCR analysis of genes associated with pancreatic endocrine development and function over 12 days of NGN3 induction post SOX17-mediated DE differentiation (indicated as day 0). Cells were cultured in regenerative MSC-CM (green lines), non-regenerative MSC-CM (blue lines), or MSC media alone (black lines) during days 3 to 12 of NGN3 induction. Levels of gene expression are compared to those from transgene-mediated cell differentiation under standard conditions (data duplicated from Figure 3.5 to facilitate direct comparison). A) markers of pancreatic endocrine differentiation, B) markers of β and/or δ cell fates, C) marker of pancreatic ductal epithelia, and D) pancreatic hormones (INS, GCG, SST) and the exocrine marker (AMY). Gene expression is corrected for input based on expression of GUSB and expressed relative to undifferentiated hESC (value set to 1). Data are presented as mean \pm SEM (n = 3). Statistical analysis was performed using two-way ANOVA with Tukey's multiple comparisons post-hoc test. X indicates significant difference ($P < 0.05$) at each time point between culture conditions.

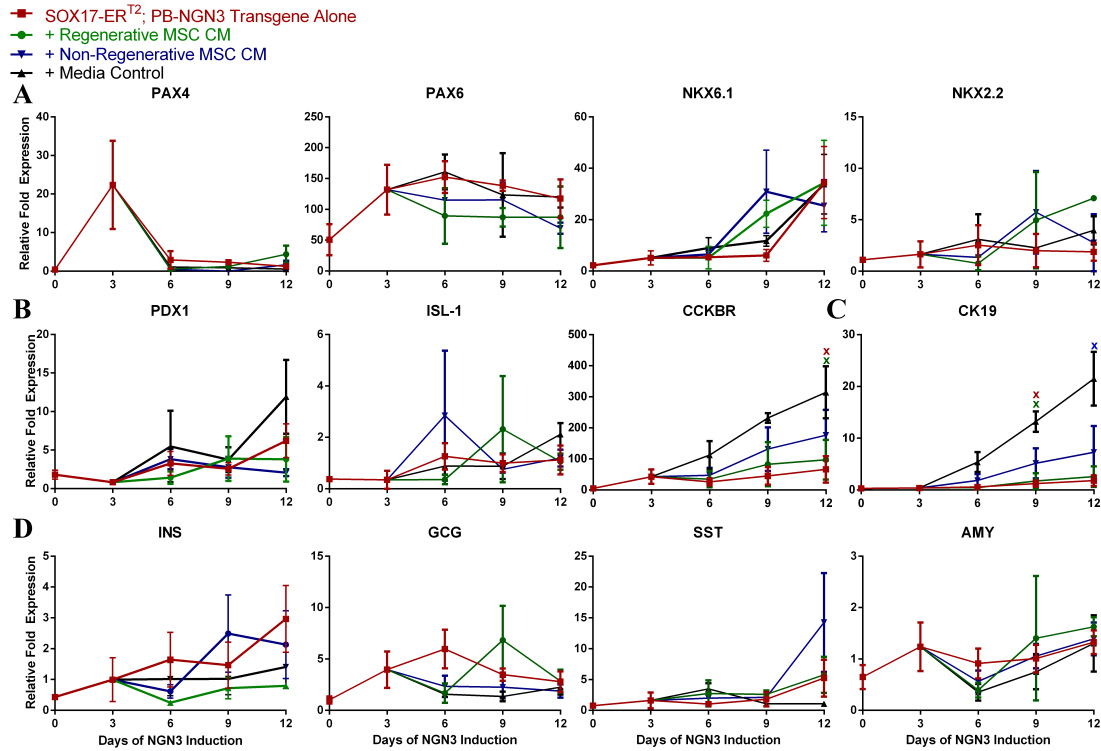


Figure 3.16. Culture of SOX17-ER^{T2}; PBNGN3^{HIGH} cells in regenerative MSC conditioned media (CM) does not promote pancreatic endocrine maturation. Real time-PCR analysis of genes associated with pancreatic endocrine development and function over 12 days of NGN3 induction post SOX17-mediated DE differentiation (indicated as day 0). Cells were cultured in regenerative MSC-CM (green lines), non-regenerative MSC-CM (blue lines), or MSC media alone (black lines) during days 3 to 12 of NGN3 induction. Levels of gene expression are compared to those from transgene-mediated cell differentiation under standard conditions (data duplicated from Figure 3.5 to facilitate direct comparison). A) markers of pancreatic endocrine differentiation, B) markers of β and/or δ cell fates, C) marker of pancreatic ductal epithelia, and D) pancreatic hormones (INS, GCG, SST) and the exocrine marker (AMY). Gene expression is corrected for input based on expression of GUSB and expressed relative to undifferentiated hESC (value set to 1). Data are presented as mean \pm SEM (n = 3). Statistical analysis was performed using two-way ANOVA with Tukey's multiple comparisons post-hoc test. X indicates significant difference (P<0.05) at each time point between culture conditions

cultured in regenerative MSC-CM from days 9-12 of NGN3 induction showed a significant upregulation of the early endocrine markers PAX6 and NKX6.1 when compared to both the non-regenerative MSC-CM and MSC media controls (**Figure 3.17**). Furthermore, NKX2.2 expression was significantly higher in islet regenerative MSC-CM than non-regenerative MSC-CM (**Figure 3.17**). These data suggests that low NGN3 expressing cells are receptive to MSC-CM at later stages of differentiation. However, no induction of mature endocrine markers PDX1, ISL1, INS or SST was observed (**Figure 3.17**). In fact, suppression of SST expression was observed following MSC-CM exposure compared to transgene-mediated differentiation alone. This suggests that regenerative MSC-CM promotes an early pancreatic precursor fate in SOX17-ER^{T2}; PB-NGN3^{LOW} cells rather than directing functional endocrine maturation. Moreover, SOX17-ER^{T2}; PB-NGN3^{HIGH} cells did not show significant changes in the expression of pancreatic endocrine markers when exposed to regenerative MSC-CM either from days 6-12 or days 9-12 (**Figure 3.18**). Collectively these data suggest that factors secreted by islet-regenerative MSCs did not efficiently promote endocrine maturation of SOX17-NGN3 pancreatic precursors.

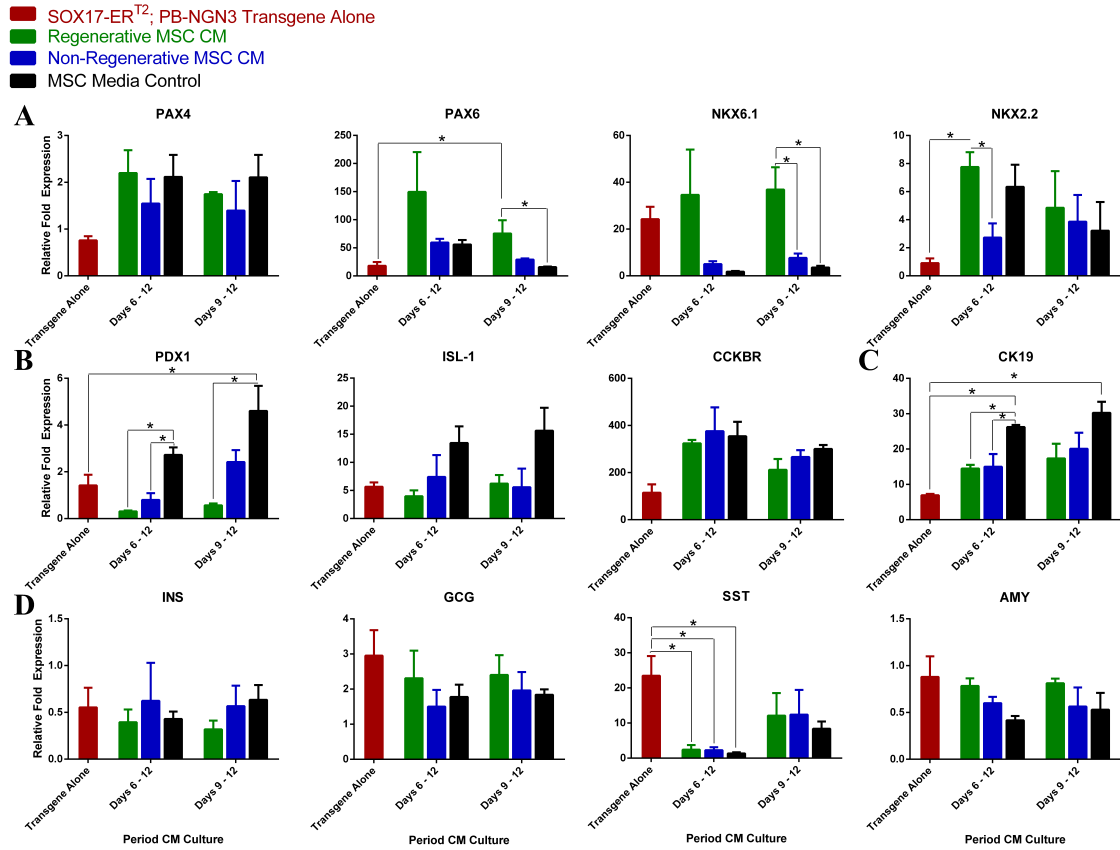


Figure 3.17. Culture in the presence of regenerative MSC conditioned media (CM) during later stages NGN3-mediated differentiation of SOX17-ER^{T2}; PB-NGN3^{Low} cells enhanced the expression of early pancreatic endocrine markers. Real time-PCR analysis of regulatory genes of pancreatic endocrine development and function: A) markers of pancreatic endocrine differentiation, B) markers of β and/or δ cell fates, C) marker of pancreatic ductal epithelia, and D) pancreatic hormones (INS, GCG, SST) and the exocrine marker (AMY) assessed following 12 days of NGN3 induction in the presence or absence of MSC-CM. Cells were cultured in regenerative MSC-CM (green lines), non-regenerative MSC-CM (blue lines), or MSC media alone (black lines) for either days 6-12 or days 9-12 of NGN3 induction. Levels of gene expression are compared to those following 12 days of NGN3 induction under standard conditions (data duplicated from Figure 3.5 to facilitate direct comparison). Gene expression is corrected for input based on expression of GUSB and expressed relative to undifferentiated hESC. Data are presented as the mean \pm SEM (n=3). Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons post-hoc test; * indicates statistical difference between the culture conditions (p<0.05).

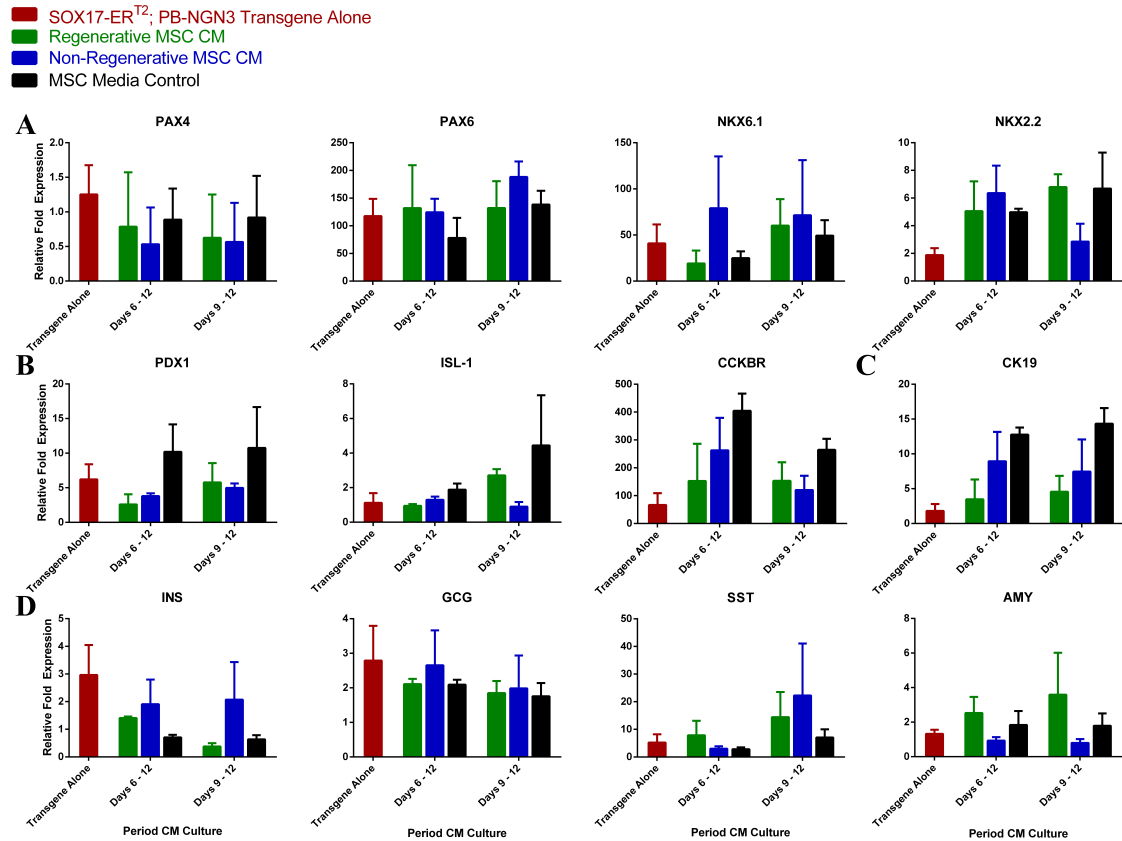


Figure 3.18. Culture in the presence of regenerative MSC conditioned media (CM) during later stages NGN3-mediated differentiation of SOX17-ER^{T2}; PB-NGN3^{HIGH} cells enhanced the expression of early pancreatic endocrine markers. Real time-PCR analysis of regulatory genes of pancreatic endocrine development and function: A) markers of pancreatic endocrine differentiation, B) markers of β and/or δ cell fates, C) marker of pancreatic ductal epithelia, and D) pancreatic hormones (INS, GCG, SST) and the exocrine marker (AMY) assessed following 12 days of NGN3 induction in the presence or absence of MSC-CM. Cells were cultured in regenerative MSC-CM (green lines), non-regenerative MSC-CM (blue lines), or MSC media alone (black lines) for either days 6-12 or days 9-12 of NGN3 induction. Levels of gene expression are compared to those following 12 days of NGN3 induction under standard conditions (data duplicated from Figure 3.5 to facilitate direct comparison).. Gene expression is corrected for input based on expression of GUSB and expressed relative to undifferentiated hESC. Data are presented as the mean \pm SEM (n=3). Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons post-hoc test; * indicates statistical difference between the culture conditions (p<0.05).

CHAPTER 4:**Discussion**

4.1. NGN3 expression in SOX17-DEPs specifies endocrine cell fate

This study demonstrates that sequential activation of SOX17 and NGN3 in pluripotent hESCs promotes pancreatic endocrine specification. During development, the induction of NGN3 induces the expression of pancreatic endocrine transcription factors including PAX4, PAX6, ISL1 and NKX6.1 [89]. Also, clonal cell lines with high levels of ectopic NGN3 expression showed a significant induction in the mRNA expression of early endocrine markers PAX4 and PAX6, and other markers implicated in β cell development and function, NKX6.1 and PDX1. In contrast, clonal cell lines with lower levels of ectopic NGN3 expression demonstrated a significant increase in the expression of known δ cell markers CCK_BR [40] and the hormone SST. However, we did not obtain convincing staining characteristic of hormone localization for SST in SOX17-ER^{T2}; PB-NGN3^{LOW} cells, suggesting that these cells are not functionally mature. In order to confirm our immunocytochemistry results and to establish that SST protein is not being translated, a more quantitative assessment of SST protein levels is required. Furthermore, induction of GCG mRNA, a marker of α cell fate, or insulin protein was not observed in either NGN3-expressing cell population. Collectively, these observations suggest that low level of NGN3 expression guides SOX17-DEPs towards pancreatic endocrine precursors that express δ cell-related genes, whereas higher levels of NGN3 expression in SOX17-DEPs promotes the differentiation of pancreatic endocrine precursors that have a mRNA expression profile more characteristic of early β cells.

Importantly, pancreatic differentiation in both clonal cell lines was not homogeneous, with clusters of differentiated cells detected throughout the heterogeneous cell populations. By immunocytochemistry, we consistently detected differentiated cells

staining in areas of high cell density, suggesting that components of the cellular microenvironment, such as paracrine signalling or cell-cell contact, may be influencing cell differentiation. Cell microenvironment plays a critical role in cell differentiation. Previous studies focusing on ectopic Pax4 expression in ES cells found enhanced cell differentiation when cells were cultured in 3D spheroids [160]. It is therefore possible that efficiency of our differentiation protocol may increase in 3D *in vitro* or *in vivo* culture conditions. It is also noteworthy that since the SOX17/NGN3 cells demonstrated continued cell proliferation, cells were passaged on day 5 of NGN3 induction in order to obtain clear immunocytochemistry images and avoid over-confluent cultures at later stages of differentiation. In contrast, cells were not passaged during the initial cell differentiation experiments where mRNA expression patterns were being assessed, meaning that cell density was significantly greater. If cell density does in fact influence cell differentiation, the difference of cell density between the two experiments may explain why we observed significant SST gene expression but we were unable to detect convincing SST immunocytochemistry.

Previous studies in mouse pancreatic adenocarcinoma (mPAC) cells and mouse pancreatic ductal epithelial cells (mPDEC) have demonstrated that the level of ectopic *Ngn3* expression influences the efficiency of differentiation towards the endocrine fate, such that higher levels of *Ngn3* expression results in greater proportions of differentiating cells than lower levels [161]. Supporting these findings, qualitative assessment of our immunocytochemistry data suggests that high NGN3 expression in SOX17-DEPs results in greater proportions of PAX6 and ISL1-positive cells than low NGN3 expression.

The expression of β cell markers after NGN3 induction in SOX17-ER^{T2}; PB-NGN3^{HIGH} cells may be due to the early induction of PAX4. During development it has been shown that PAX4 can promote the β cell fate [91], and repress the α -cell lineage [162] in pancreatic endocrine progenitors. Apart from β cell fate specification, PAX4-positive progenitors have been also been implicated in δ cell differentiation during development [91]. However, we did not observe significant induction of PAX4 levels in SOX17-ER^{T2}; PB-NGN3^{LOW} cells which showed a significant increase in the expressions of δ cell-related genes upon NGN3 induction. Our findings are supported by studies showing that PAX4 is not required for δ cell specification. Indeed, mice lacking expression of both *Pax4* and the α cell fate specification regulator *Arx* showed complete depletion of α and β cells, resulting in a δ cell dominated pancreas [163]. This suggests that Pax4 may serve more specifically to promote the β cell fate. Our study supports these findings by showing that significant induction of PAX4 expression is not necessary for the induction of SST-expression in the SOX17-ER^{T2}; PB-NGN3^{LOW} clone.

Although SOX17-ER^{T2}; PB-NGN3^{HIGH} cells showed a significant induction in the mRNA expression of a known β cell marker PDX1, we did not observe a significant induction of INS mRNA or protein. This suggests that these cells may represent β cell precursors that are phenotypically and functionally immature. SOX17-ER^{T2}; PB-NGN3^{LOW} cells showed significant SST gene induction. Importantly, in both cell lines we observed a lack of NKX2.2 mRNA induction, which is an important transcription factor for terminal differentiation of β cells [98] and repression of SST gene expression during development [99]. The lack NKX2.2 induction in both clones may help to explain why we observed more efficient induction of genes associated with δ cells than β cells.

Although PDX1 is commonly used as a β cell marker [164,165], it is also expressed in the developing gastric enteroendocrine cells [82,83]. Similarly, SST expression is not pancreas-specific but is also found in endocrine cells of the stomach and the intestine [166,167]. Therefore, we cannot rule out the possibility that NGN3 induction in DEPs may be promoting the differentiation of other endoderm-derived endocrine cell lineages. Future studies should focus on global mRNA expression analysis to better assess the effects of NGN3-mediated differentiation on DEPs.

We detected some discrepancies when comparing our immunocytochemistry results with mRNA expression analysis. For instance, we detected PAX6 and ISL1 protein expression in SOX17-ER^{T2}; PB-NGN3^{LOW} cells but we did not observe significant induction in their mRNA expression during the course of NGN3 induction. Also, for SOX17-ER^{T2}; PB-NGN3^{HIGH} cells we detected a subset of cells with ISL1 protein induction despite the lack of a significant change in mRNA expression. The technical limitations associated with quantifying gene expression within a heterogeneous population versus being able to identify and image subpopulations of cells expressing candidate protein by immunostaining could explain these discrepancies. In our immunocytochemistry data we observe a mixed population of protein-positive and negative cells. Since our mRNA analysis was performed on the total population of cells it is likely that the mRNA expression of the population was not significant due to a large proportion of non-differentiated cells. Furthermore, poor correlation of mRNA and protein expression has been previously noted in literature. In fact, mRNA expression and protein levels have a correlation coefficient of less than 0.4 [168]. Similar to our findings, several studies have shown that protein levels do not correlate with transcript levels [169-

172]. For instance, Tian *et al.* measured the expression of 425 proteins and mRNA in multipotent mouse hematopoietic progenitor EML cells and their differentiated progeny. This study mapped the protein expression to their corresponding mRNA expression and showed that 35% of the genes investigated had significantly upregulated protein levels but the mRNA expression remained unaltered [169]. Such discrepancies are primarily due to post-transcriptional and translational regulation [168].

4.2. The level of NGN3 influences endocrine cell fate determination.

Previous studies have shown that alterations in *Ngn3* expression *in vivo* have considerable effects on determination of endocrine *versus* exocrine cell fates. Endocrine progenitors expressing reduced levels of *Ngn3* adopted pancreatic ductal or acinar fates while high levels of *Ngn3* expression was reported to be critical for endocrine commitment from multipotent pancreatic progenitors [173]. In addition, studies have shown that the level of *Ngn3* expression has a considerable impact on the expression of downstream factors regulating endocrine differentiation. The transduction of a *Ngn3* retroviral transgene in mouse pancreatic ductal epithelial cells (mPDEC) demonstrated that *Pax4* expression is linearly dependent on the level of *Ngn3* expression [161]. We observed a similar correlation between the level of ectopic NGN3 expression with the mRNA expression of the early endocrine TFs PAX4 and PAX6 in SOX17-DEPs.

We suggest that the difference in the induction of early endocrine transcription factors PAX4 and PAX6 influences the difference observed in the differentiation of high and low NGN3 expressing SOX17-DEPs. Previous studies have suggested that Pax4 and Pax6 orchestrate β cell differentiation, and that diminished levels of either of these

factors, as observed in *Pax4*-null and *Pax6*-null mice, leads to the development of ghrelin positive ϵ cells [174]. Based on our data, we suggest that the specific level of PAX4 and PAX6 expression induced differentially regulates endocrine fate specification, where lower levels of expression direct δ cell specification, whereas higher levels of expression direct β cell specification.

Collectively, our data along with previous studies show that the level of NGN3 expression significantly impacts the expression of downstream endocrine transcription factors. These findings may be related to the short half-life of the Ngn3 protein associated with post-translational ubiquitin-mediated degradation [175], and thus high levels of transcription and protein production may be critical for maintaining sufficient bioavailability for transcriptional activity.

In order to improve our understanding of the effects of NGN3 expression on endocrine differentiation, future studies should more thoroughly characterize the phenotype of SOX17-ER^{T2}; PB-NGN3^{LOW/HIGH} cells by whole transcriptome analyses. Furthermore, to assess the biological activity of NGN3, chromatin immunoprecipitation with parallel DNA sequencing (ChIP-sequencing) could be performed for cell lines with different levels of NGN3 expression to identify direct NGN3 targets. This global characterization would provide a better understanding of the interplay between NGN3 DNA binding, dose and bioavailability.

Considering that previous studies aimed at generating pancreatic cells from pluripotent human cells show an improvement in the efficiency of endocrine differentiation after transplantation *in vivo* [130], it would also be informative to compare the *in vivo* differentiation of SOX17-ER^{T2}; PB-NGN3^{LOW/HIGH} cells using standard

teratoma assays or recently developed protocols for intra-pancreatic injection in NOD-SCID mouse models after streptozotocin-induced deletion of endogenous β cells [121,126].

4.3. Islet-regenerative MSCs conditioned media did not promote maturation of NGN3-positive endocrine precursors

The current study also aimed to capitalize on previous studies by our group showing that certain human bone-marrow derived MSC samples were able to induce new islet formation following transplantation into STZ-treated NOD/SCID mice [121,126,146], while other BM MSC samples were not [121]. We hypothesized that paracrine factors secreted by islet regenerative MSC samples would promote the maturation of SOX17-NGN3 pancreatic endocrine precursors towards the β cell fate. However, our data shows that islet-regenerative MSC-CM did not direct NGN3-expressing precursors to a mature phenotype *in vitro*. One potential explanation may be that additional factors or cell-cell contact present *in vivo* may be required to direct terminal differentiation. This is supported by previous studies that demonstrate the generation of insulin secreting cells from hESC only happens following *in vivo* maturation of cells [130]. It is also possible that the NGN3-positive precursors generated in these studies may not be responsive to islet regenerative MSC secreted factors. Previous studies have shown that NGN3-positive progenitors were not detected at specific time points during MSC-induced islet-regeneration [126], suggesting that paracrine factors secreted by regenerative MSCs may not act through an NGN3-positive

precursor cell or NGN3 expression was not induced in endogenous adult pancreatic cells as part of the islet-regenerative process.

Furthermore, other studies have also suggested that CK19-positive ductal pancreatic progenitor cells may represent primary effectors implicated in adult islet neogenesis [176,177]. Indeed, MSC-induced islet formation was associated with ductal structures, and these small neoislets contained CD19⁺ cells as evidence of ductal cell involvement in the regenerative process [121,126]. Alternatively, MSC secreted factors may act on a more differentiated endocrine precursor cell that has already reduced NGN3 expression and gained CK19 expression. Interestingly, the SOX17-ER^{T2}; PB-NGN3^{LOW} cells, which demonstrated induction of early pancreatic endocrine markers after culture with islet-regenerative MSC-CM during the later stages of NGN3 induction (days 9-12), were also found to express CK19 at this later stage of differentiation. In contrast, SOX17-ER^{T2}; PB-NGN3^{HIGH} cells that do not express CK19 did not show changes in pancreatic endocrine markers expression following exposure to islet-regenerative MSC-CM. Therefore, similar to the *in vivo* tissue repair response, CK19-expressing SOX17-ER^{T2}; PB-NGN3 cells may be more receptive to paracrine factors secreted by islet-regenerative MSCs. However, it is possible that CK19 does not co-localize with other endocrine markers (PAX6, ISL1), and may represent a distinct cell population. Protein co-localization studies should be performed to understand the context of CK19 expression during NGN3-mediated differentiation of DEPs.

Importantly, the complete mechanism associated with MSC-induced islet-regeneration *in vivo* has yet to be determined; however the results from the current study suggest that the factors that promote islet-regeneration *in vivo* may be distinct from the

factors required to promote functional maturation of hESC-derived NGN3-positive endocrine precursors. For instance, islet-regenerative MSCs have been shown to possess pro-angiogenic and anti-inflammatory potential [126], that may be important in inducing islet-regeneration in whole organ systems *in vivo*, but may not be relevant to promote pancreatic endocrine maturation of DEPs. Therefore, we propose that better understanding of the mechanisms by which islets are formed during development and during MSC-induced islet regeneration is required before achieving the ultimate goal of generating an unlimited number of functional β cells from pluripotent sources.

4.4. Limitations of the Study and Future Directions

There are several potential limitations associated with the use of conditioned media to recreate the MSC-mediated islet-regenerative microenvironment. First is the likelihood that short-term factors and cytokine secreted into the CM were degraded due to freezing of the conditioned media prior to experimentation. Alternatively, direct cell-cell contact between MSC and pancreatic progenitors may be required for the islet neogenesis characterized *in vivo*. As a consequence, a complete recreation of *in vivo* regenerative microenvironment may not be possible using CM. In order to tackle these limitations, future experiments should focus on direct co-culture of MSCs and putative endocrine precursors to better assess the effects of islet-regenerative factors on maturation of endocrine precursors. Finally, the *in vitro* monolayer culture used in these studies does not mimic the *in vivo* 3D environment that may contribute to the maturation of SOX17-NGN3 pancreatic precursors. Therefore, these experiments should be repeated *in vivo* using co-transplantation of regenerative MSCs and NGN3-positive precursors.

This will test the effects of MSC-maturation signals in a 3D *in vivo* environment enabling a direct comparison with the *in vitro* experiment conducted in this study.

Additionally, the protocol developed for transcription factor-mediated pancreatic endocrine specification from DEPs skips two key developmental stages that follow endoderm specification during embryonic development: primitive foregut gut specification [61] and PDX1-mediated pancreas specification [76], which occur before the initiation of NGN3-mediated endocrine differentiation [62]. Therefore, future experiments should investigate the effects of sequential induction of PDX1 then NGN3 in SOX17-DEPs in order to formulate a more efficient transcription factor based protocol for differentiating stable and mature endocrine cells from pluripotent stem cells.

4.5. Conclusion and Summary

The current study demonstrated that NGN3 activation after SOX17-mediated DEP specification in hESC promotes step-wise induction of transcription factors important for pancreatic endocrine differentiation. We also show NGN3-mediated differentiation to be dose-dependent, with low levels of NGN3 expression promoting δ cell-like characteristics, and high expression of NGN3 promoting the induction of some β cell-like characteristics. Moreover, we found that these endocrine progenitor populations were not responsive to signals secreted by islet-regenerative MSCs, suggesting that microenvironmental regulators of islet regeneration after injury and the normal development of NGN3-positive progenitor cells may be distinct.

CHAPTER 5:**References**

5.1. References

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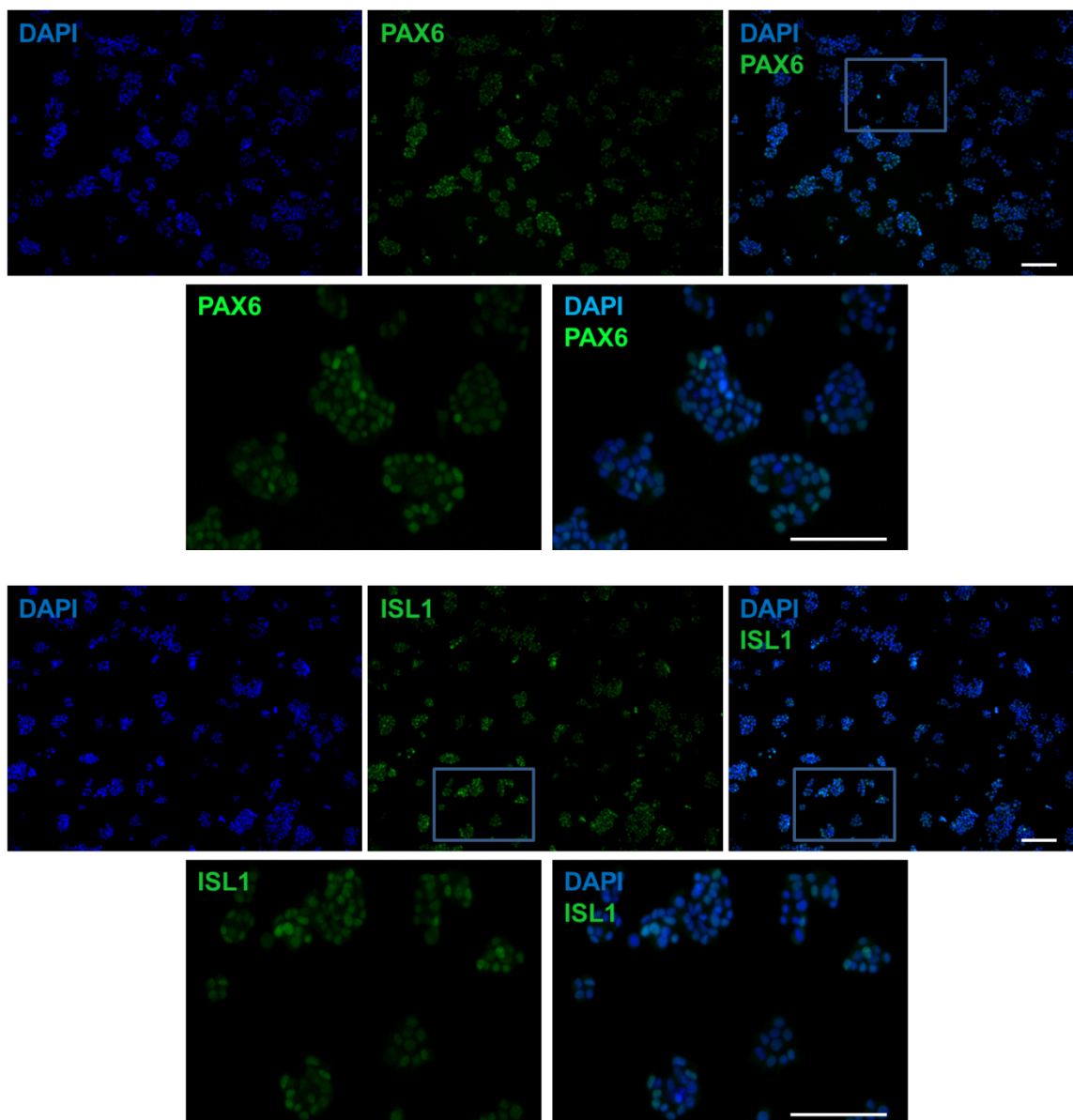
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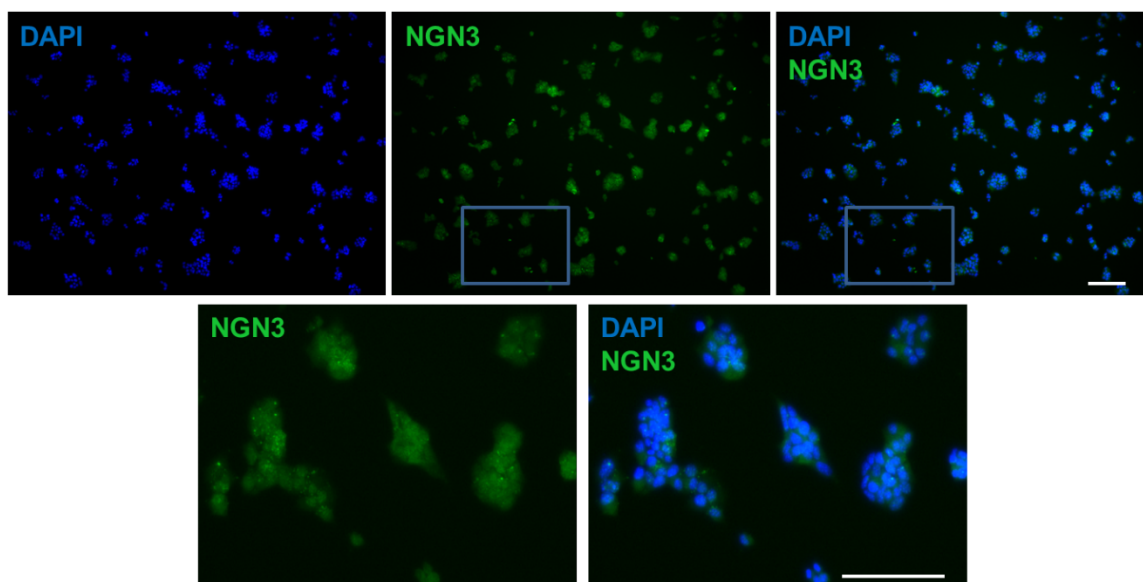
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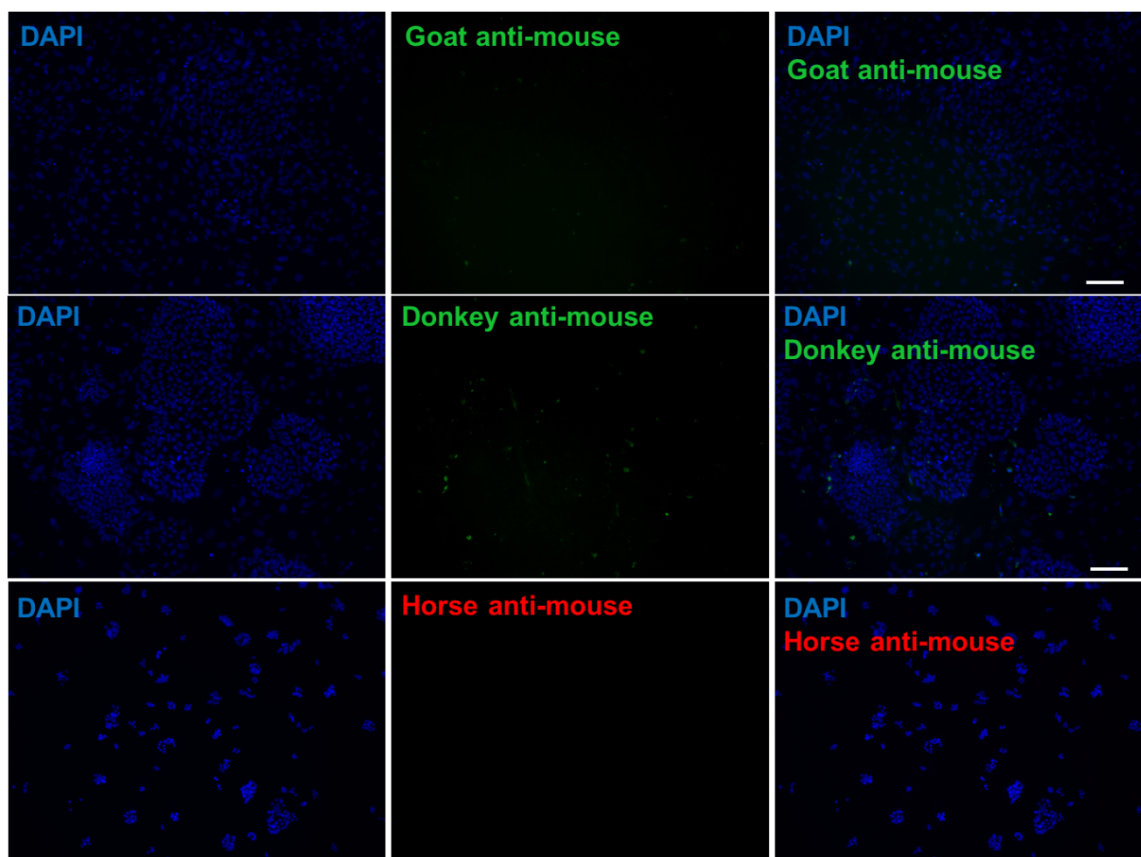
Appendix

Appendix 1. Immunocytochemistry positive control experiments. Staining of β TC6 cells (an insulin-secreting cell line derived from transgenic mice expressing the large T-antigen of simian virus 40 (SV40) in pancreatic β cells as a positive control for endocrine markers: PAX6, ISL1 and NGN3. Scale bar equals 100 μ m.





Appendix 2. Immunocytochemistry negative control experiments. Staining only with secondary antibodies used in this study. Scale bar equals 100 μm .



Curriculum Vitae

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NGN3 expression in DEPs specifies pancreatic endocrine fate.
Shah A, Brooks C, Hess DA, Seguin CA. Presented at Diabetes
Research Day, Western University, London ON., 2013.

NGN3 expression in DEPs specifies pancreatic endocrine fate.
Shah A, Brooks C, Hess DA, Seguin CA. Presented at London
Health Research Day, London, ON., 2014.